Biodegradation of sodium benzoate by *Pseudomonas* biofilm consortium in a fluidized bed bioreactor

MSc Thesis

By

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DECLARATION

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I am also thankful to my grandmother and my aunt for taking care of me and encouraging me through it all.

Dedication

This thesis is dedicated to my parents who have passed away especially my mother, who, always believed in me.

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LIST OF ABBREVIATIONS

PAHs	Polycyclic Aromatic Hydrocarbons
GAC	Granular Activated Carbon
CFU	Colony Forming Unit
OD	Optical Density
NA	Nutrient Agar
BA	Benzoate Agar
SEM	Scanning Electron Microscopy
AOPs	Advanced Oxidation Processes
COD	Chemical Oxygen Demand
BTEX	Benzene, Toluene, Ethylbenzene and Xylenes
EPS	Extracellular Polymeric Substances
FBBR	Fluidized Bed Biofilm Reactor
CSTR	Continuous Stirred Tank Reactor
TBR	Trickling Bed Reactor
ALR	Airlift Reactors
EGSB	Expanded Granular Sludge Blanket
USB	Upflow Sludge Blanket
IC	Internal Circulation
N	Nitrogen
P	Phosphorus

Abstract

Many strains of Gram-negative bacteria, such as *Pseudomonas*, are able to utilize a variety of unusual chemicals, including a wide range or aromatic hydrocarbons and their derivatives for growth. Bacteria with the potential to degrade sodium benzoate were isolated, identified and grown as biofilms on sodium benzoate in a laboratory-scale fluidized bed biofilm bioreactor. Four *Pseudomonas* strains identified as *P. aeruginosa* (BDS2) *P. putida* (BDS1 and GR1) and *Burkholderia cepecia* (GR3FAR) were used in a laboratory-scale FBBR together with two *Bacillus* strains - *Bacillus macroides* (SBSY4) and *Bacillus simplex* (MAR). Sodium benzoate biodegradation capacities of these species were compared under batch and continuous operations. Biofilm and planktonic bacterial growth dynamics were monitored by plate counts, and optical density measurements (230nm) determined benzoate biodegradation. Overall, higher attached and planktonic bacterial counts were determined under batch compared to continuous mode. In addition to this, the ability of attached cells to use sodium benzoate as their sole carbon source was compared to their suspended counterparts in a batch system. There were more attached counts compared to suspended cells and attached cells apparently degraded sodium benzoate better than planktonic cells. Similarly, higher rates of benzoate depletion were found to occur under batch compared to the continuous system. It thus appeared that more cell growth implied more substrate consumption. SEM showed attached cells and microcolonies of all the isolates on GAC, indicating their biofilm-forming abilities.

Key words: Biodegradation, sodium benzoate, *Pseudomonas*, biofilm, batch, continuous.

Chapter 1

Literature review

Introduction

1.1 Wastewater treatment

The term, "wastewater", represents all domestically, agriculturally and industrially generated wastewater effluents, and its treatment is important for sustainable management of water resources (Engin & Demir, 2006). Wastewater treatment proceeds through the following three phases (Massé & Massé, 2000): (i) Primary treatment involving the physical removal of large solids by grates, screens, and settling tanks, resulting in approximately 60 % removal of suspended solids from the wastewater. (ii) Secondary treatment involves the removal of organic matter using lagoons, activated sludge systems, extended aeration, oxidation ditches and sequencing batch reactors. (iii) Tertiary treatment involves the removal of N or P or suspended solids (Mittal, 2006) (Figure 1.1).

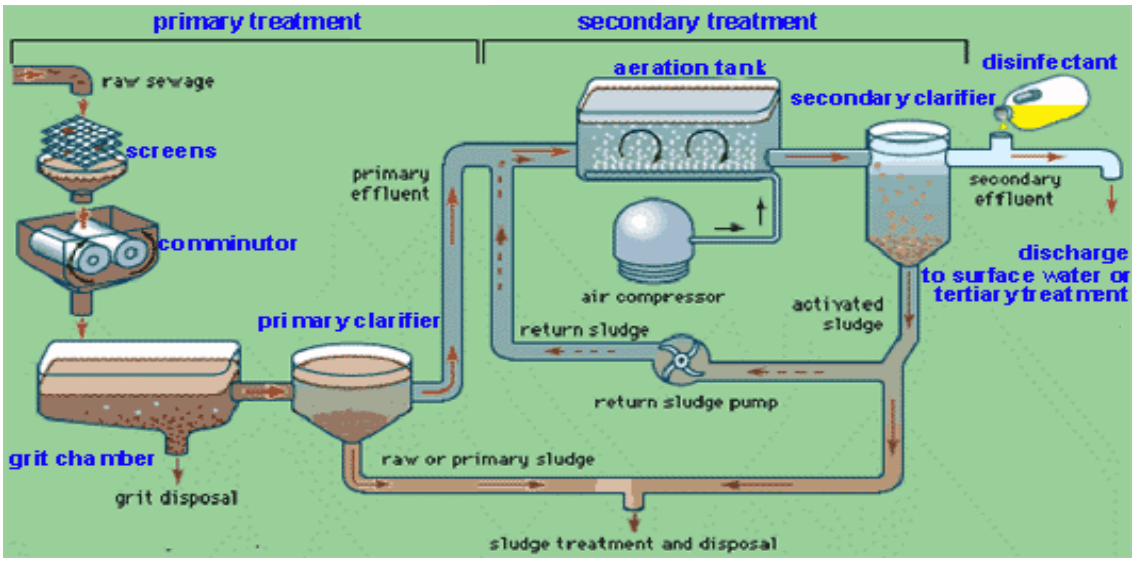

Figure 1.1 showing all the steps involved in wastewater treatment (Earthpace Resouces, 2007).

Different treatment methods are used for different industrial wastewaters. For example, for oily wastewaters, processes such as gravity separation (the primary treatment), dissolved air flotation, de-emulsification, coagulation and flocculation, and membrane processes such as microfiltration (MF) (Cheryan & Rajagopalan, 1998) have effectively been used.

In treating wastewaters from textile industries, combined treatment methods, such as chemical coagulation–flocculation, chemical oxidation, activated-carbon adsorption and anaerobic biological treatment, usually with a secondary activated-sludge treatment step, are normally used (Rott & Minke, 1999).

Different physical, chemical, and biological methods, such as activated carbon adsorption, chemical oxidation, and aerobic/anaerobic biological degradation are used for removal of chlorophenols from wastewater (Atuanya *et al*., 2000; Jung *et al*., 2001). Treatment technologies available for phenolic wastes are physical, chemical, biological and electrochemical processes.

With the increasing diversity of industrial waste products, many of which (usually), may also contain recalcitrant hydrocarbon pollutants, industrial wastewater effluents are becoming more complex (Alinsafi *et al*., 2006). Therefore introduction of new technologies to degrade these molecules is vital (Gogate & Pandit, 2003).

The new technologies include advanced oxidation processes (AOPs), such as cavitation, which is generated using constrictions, such as valves (Adewuyi, 2001), photocatalytic oxidation using UV light/sunlight (Bhatkhande *et al*., 2002), or Fenton's

chemistry/ reaction using reactions between Fe ions and hydrogen peroxide (Bigda, 1996), and chemical oxidation involving the use of oxidizing agents, such as ozone and hydrogen peroxide (Gogate & Pandit, 2003). AOPs have the potential to degrade the new toxic chemicals, bio-refractory compounds, pesticides and organic compounds either partially or fully, but most importantly under ambient conditions (Gogate & Pandit, 2003).

In the case of the chemical and textile industries, wastewater effluents may have high chemical oxygen demand (COD) levels consisting of xenobiotic aromatic hydrocarbons that are recalcitrant with regard to their biodegradability by normal aerobic or anaerobic bacterial metabolic pathways (Dantas *et al*., 2006). Even so, many bacterial species exist which have evolved an amazing ability to metabolize almost any organic carbon substrate that may be present in wastewater effluents (Hutchinson & Robinson, 1988).

Many sewage works or wastewater bioremediation plants depend on this microbial capacity for the mineralization of organic substrates. According to Bergamasco (1996), cell respiratory or catabolic pathways is/are the main mechanism/s involved in the biodegradation carried out by microorganisms in aerobic processes (Bergamasco, 1996). These processes use oxygen as the main electrons acceptor, thereby causing the oxidation of organic compounds. It also transforms complex molecules into simpler and more stable ones (Lehninger, 1976).

1.2 Aromatic hydrocarbons

Aromatic hydrocarbons are hydrocarbons that contain one (monoaromatic) or more (polyaromatic) benzene rings (Korenaga *et al*., 2000). They include benzene and the alkyl derivatives of benzene, represented as a six sided ring containing alternating single and double bonds, or with a circle in the middle (Morrison & Boyd, 1973). They are highly hydrophobic, with a low water solubility and biodegradability (Shemer & Linden, 2007). Most of these compounds may be formed during incomplete combustion of organic materials (WHO, 2005). They are also found as food contaminants during food processing (Tfouni & Toledo, 2006).

Polycyclic aromatic hydrocarbons (PAHs) are of most environmental concern due to their toxicity, low volatility, resistance to microbial degradation, and high affinity for sediments (Lovley *et al*., 1994; Tfouni & Toledo, 2006). Some of them are also carcinogenic and bioaccumulate in aquatic organisms (Shemer & Linden, 2007) (Figure 1.2.).

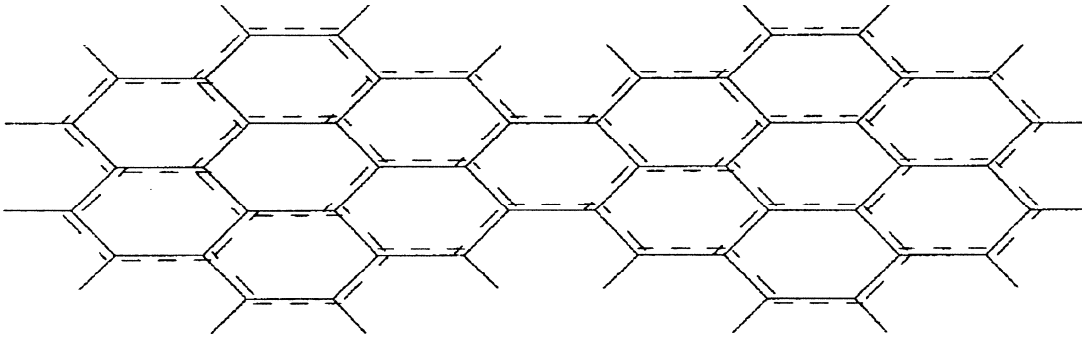

Figure 1.2 An example of PAH- Dicoronene - largest aromatic hydrocarbon detected in coal-tar pitch. (Marzec, 2000).

Wastewater effluents from petrochemical industries contain a wide range of aromatic compounds, for example, benzene, toluene, ethylbenzene, xylene, styrene, and naphthalene (Gómez *et al*., 2001). Wastewaters containing phenolic pollutants are also generated from the paint, pesticide, coal conversion, polymeric resin, petroleum and petrochemicals industries (Aksu & Yener, 2001; Dabrowski *et al*., 2005). Other industries that produce similar phenolic pollutants are the pharmaceutical, coke, polymer, paint and dye industries (Low *et al*., 1988).

Aromatic hydrocarbons can be removed from the environment by processes such as volatilization, photo- and chemical oxidation, adsorption to soil particles, leaching, bioaccumulation and biodegradation (Shemer & Linden, 2007). Bioremediation has been found to derive efficiency and economy advantages (Singh *et al*., 2006).

a) Aromatic hydrocarbon degrading organisms

Hydrocarbon-degrading microorganisms are normally found in marine, freshwater, and soil ecosystems (Atlas, 1986). Isolation of high numbers of hydrocarbon-degrading microorganisms from a certain environment usually means that the isolated organisms are the degraders within that environment (Okerentugba & Ezeronye, 2003).

A number of soil inhabiting microorganisms have the ability to degrade and utilize as carbon substrates, various aromatics including toluene (Chang *et al*., 1993), benzene (Kukor & Olsen, 1991), phenol (Murray & Williams, 1974; Hutchinson & Robinson, 1988), chlorobenzene (Haigler *et al*., 1992), and nitrotoluene (Haigler *et al*., 1994). This ability is exhibited by a wide variety of bacterial and fungal genera (Leahy & Colwell, 1990).

Among fungi, aromatic degrading species include *Aspergillus, Penicillium, Fusarium, Amorphoteca, Neosartorya, Paecilomyces, Talaromyces* and *Graphium* (Bumpus *et al*., 1985). The predominant bacteria of polluted soils belong to a spectrum of genera (Table 1.1) (Fetzner & van der Meer, 2000). Bacterial species with aromatic degrading capacities (Whited & Gibson; 1991, Fetzner & van der Meer, 2000) have been found in the genera *Gordonia, Brevibacterium, Aeromicrobium, Dietzia, Burkholderia/ Pseudomonas, Mycobacterium* and *Bacillus* (Chaillan *et al*., 2004) with *Pseudomonas putida* being the best characterized (Nichols & Harwood, 1995; Reardon *et al*., 2000).

Table 1.1 Bacteria predominantly found in soils polluted with aliphatic and aromatic hydrocarbons, polycyclic aromatic hydrocarbons, and chlorinated compounds according to Fetzner & van der Meer (2000) (Fetzner & van der Meer, 2000).

Gram-Negative Bacteria	Gram-Positive Bacteria
Pseudomonas spp.	*Nocardia* spp.
Acinetobacter spp.	*Mycobacterium* spp.
Alcaligenes sp.	*Corynebacterium* spp.
Flavobacterium/ *Cytophaga* group	*Arthrobacter* spp.
Xanthomonas spp.	*Bacillus* spp.

b) Aromatic hydrocarbon degradation by *Pseudomonas*

The genus *Pseudomonas* consists of gram-Negative, rod shaped aerobic bacteria with polar flagella and has been isolated principally from soils. Many Pse*udomonas* species have been reported to degrade a variety of aromatic hydrocarbons (derived from) associated with oil, petroleum and pesticide pollutants (Fujii *et al*., 1997). These include the BTEX (benzene, toluene, ethylbenzene and xylenes) group of aromatic hydrocarbons. Therefore this group of bacteria has been identified as important bioremediation agents because of their capacity to degrade or metabolize most of the environmentally toxic BTEX pollutants (Bettman & Rehm, 1984).

Pseudomonas degrades aromatic hydrocarbons by converting them into catechol by either a *meta* ring cleavage pathway or an *ortho* ring cleavage pathway (Harwood & Parales, 1996). This can be achieved aerobically by oxidation of the alkyl side chain (Burlage *et al*., 1989) or aromatic ring via a mono-oxygenase (Kahng *et al*., 2001) or a dioxygenase attack (Zylstra & Gibson, 1989). Catechol 2, 3-dioxygenases (C23O) catalyses the ring cleavage step after which the structure is further degraded into Krebs cycle intermediates (Harayama & Rekik, 1993; Reardon *et al*., 2000) (Figure 1. 3).

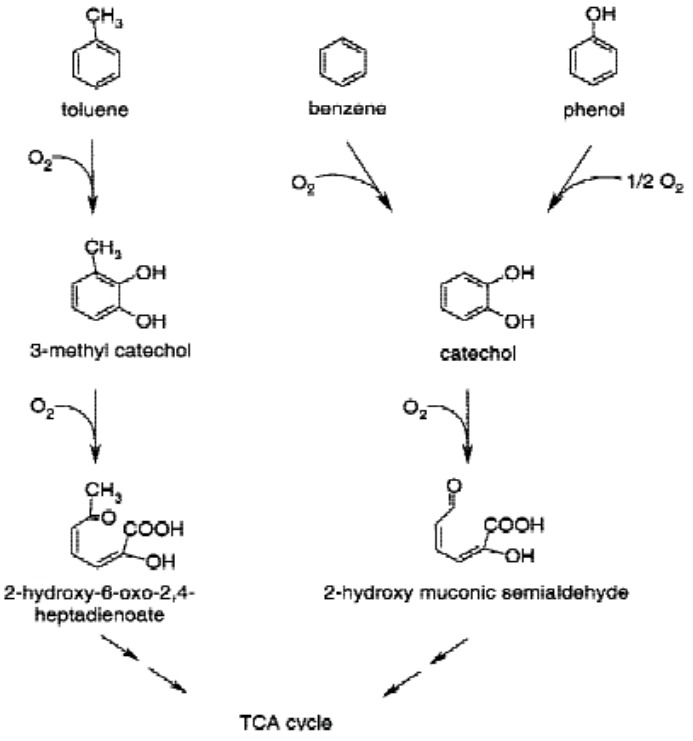

Figure 1.3 Catabolic pathways for toluene, benzene, and phenol in *P. putida* F1 (Reardon *et al*., 2000).

1.3 Benzoate as a model biodegradation substrate

Various microorganisms are capable of degrading aromatic compounds, such as benzoate under aerobic and anaerobic conditions (Kukor & Olsen, 1991). Aerobic metabolism of aromatic compounds, such as benzoate, has been studied in considerable detail in various microorganisms (Stanier & Ornston, 1973). Benzoate (or benzoyl-CoA) is the most common intermediate in anaerobic metabolism of aromatic compounds and recently, benzoate has been reported to be an intermediate of anaerobic benzene biodegradation (Caldwell & Suflita, 2000). Its biodegradation in *Pseudomonas* has been reported to proceed strictly *via* the *ortho* cleavage pathway (Feist & Hegeman, 1969) but other studies have shown the possibility of both *meta* and *ortho* pathways (Nakazawa & Yokota, 1973).

P. putida has been reported to be able to convert benzoate to catechol using chromosomally encoded enzymes. Catechol is then further degraded to trichloroacetic acid cycle intermediates by an *ortho* ring cleavage pathway (Harwood & Parales, 1996). Benzoate has been shown to be an inducer of the synthesis of the TOL plasmid-encoded enzymes of the *meta* fission pathway (Franklin *et al*., 1981) as well as synthesis of the chromosomally encoded enzymes that convert benzoate to catechol (Figures 1. 4 and 1.5).

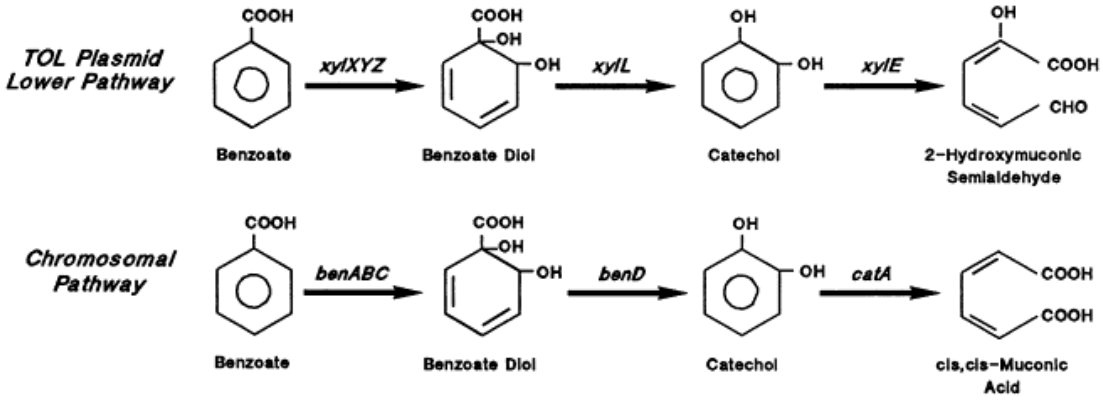

Figure 1.4 Chromosomally and TOL plasmid encoded genes for benzoate catabolism. Toluene dioxygenase (*xyl*XYZ) is among enzymes encoded by TOL plasmid and benzoate dioxygenase (*ben*ABC) include chromosomally encoded enzymes (Jeffrey *et al*., 1992).

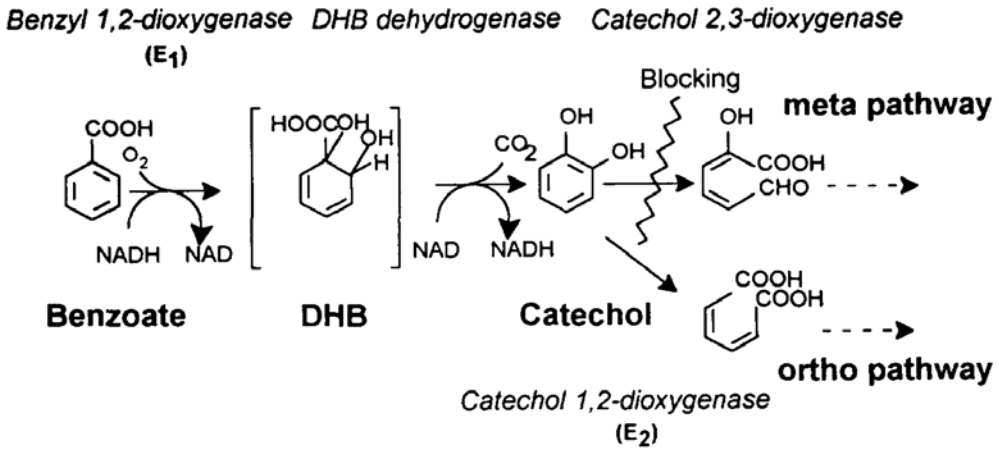

Figure 1.5 Benzoate degradation in *Pseudomonas* species. In *Pseudomonas putida*, the TOL plasmid pWW0 encodes all the enzymes responsible for the degradation of toluene and xylene. The genes encoding these enzymes are organized into two operons – *meta* and *ortho* operons. The *meta* operon contains the genes responsible for the degradation of benzoates and toluates (Jeong & Young, 1997).

1.4 Biofilms as bioremediation systems

Biofilms can be defined as complex aggregations of microorganisms growing on a solid substrate (Qureshi *et al*., 2005). Biofilm formation involves four steps: initial attachment, irreversible attachment by the production of extracellular polymeric substances (EPS), early development, and maturation of biofilm architecture (Stoodley *et al.,* 2002). A biofilm starts with attachment of a free floating cell to a surface (Qureshi *et al*., 2005). It is after this initial attachment step that the cell will form an association (temporary) with the surface (Watnick & Kolter, 2000), after which it can detach from the surface or attach irreversibly by producing EPS (Donlan & Costerton, 2002; Qureshi *et al*., 2005) (Fig 6). EPS benefits the cell in many ways. It binds the cell irreversibly to the surface and acts as a protective barrier to phagocytes, bacteriocides and compounds that could be toxic to the cells (Gilbert *et al*., 1997).

As a biofilm develops and matures it develops water channels (Qureshi *et al*., 2005). Highly permeable water channels interspersed through out the biofilm in the areas surrounding the microcolonies and provide an effective means of exchanging nutrients and metabolites with the bulk aqueous phase, enhancing nutrient availability as well as removal of potentially toxic metabolites (Sturman *et al*., 1995).

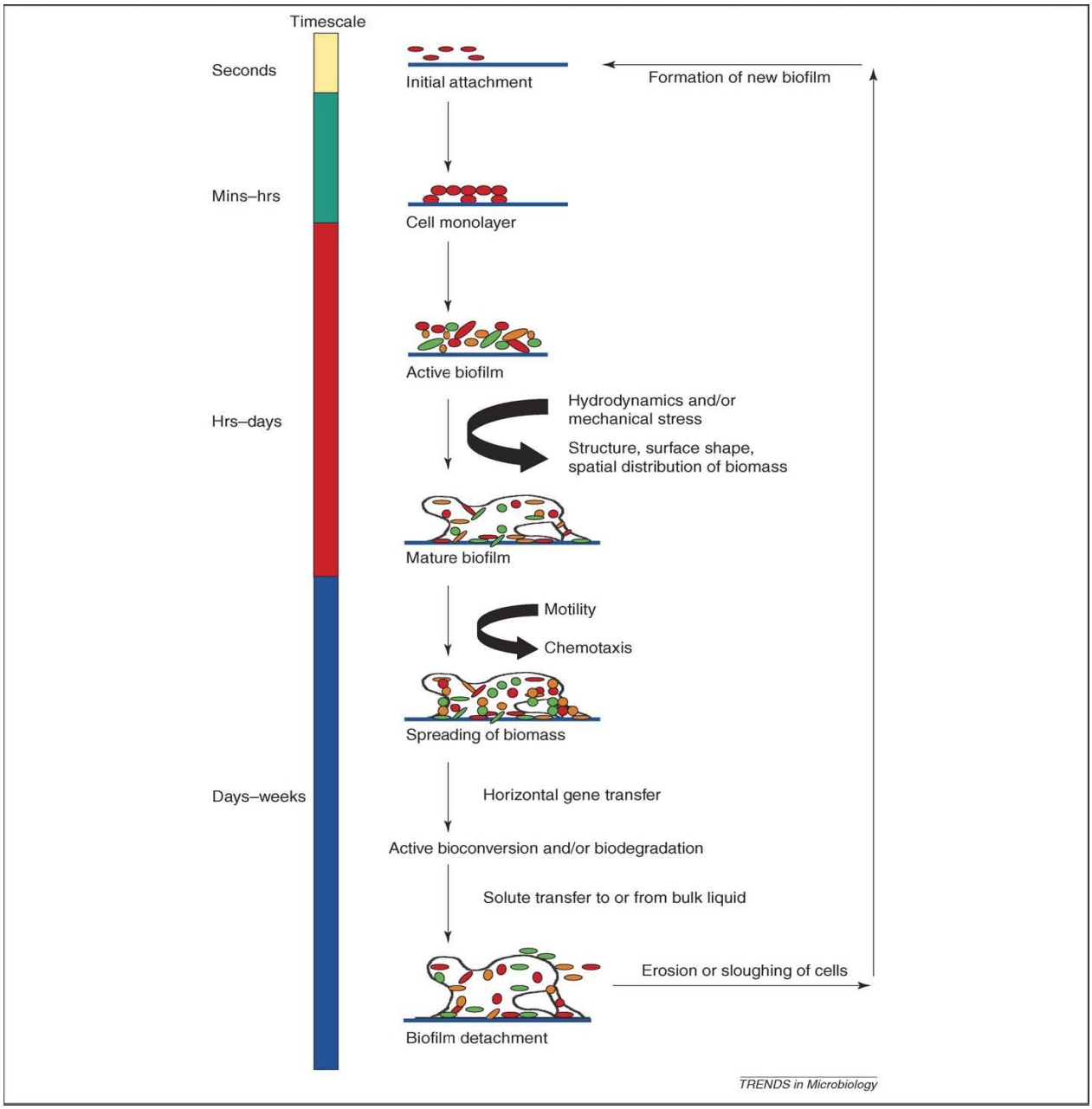

Figure 1.6 A diagrammatic representation of all the steps in biofilms formation. The first step is attachment, which is followed by maturation and finally detachment. The motile cells in the mature biofilms undergo chemotaxis which results in spreading of biomass and higher rates of gene transfer (Singh *et al*., 2006).

There are several factors that affect the rate at which biofilms form (Qureshi *et al*., 2005). These include, hydrodynamics of the fluid (Kugaprasatham *et al.*, 1992; Allan *et al*., 2002)**,** the nature and type of the surface or environment (fine vs. rough) (Characklis *et al*., 1990), hydrophobicity (e.g. Teflon vs. metal/glass) (Dalton *et al.*, 1994; Donlan, 2002), species composition (Lawrence *et al.*, 1991), temperature which affect EPS production (Annachhatre & Bhamidimarri, 1992) and nutrient availability (Cowan *et* al., 1991; Huang *et al.*, 1994).

Biofilms have been successfully utilized in bioremediation of toxic compounds. Their success lies mainly on what is called syntrophism (Davey & O'toole, 2000); a phenomenon discovered by Bryant and colleagues in 1967 (Bryant *et al*., 1967) .This has been well illustrated in methanogenic degradation (Schink, 1997). In this case the fermenting bacterium only grows on ethanol at a very low hydrogen partial pressure, conditions provided for by the methanogen bacterium, and the methanogen obtains its energy from the fermentative bacteria (Davey & O'toole, 2000). In this way both bacteria exhibit a metabolic activity that neither one could accomplish on its own. In degradation of aromatic hydrocarbons, syntrophic interactions have been confirmed to exist only for benzoate, gentisate, and hydroquinone (Schink, 1997).

1.5 Biofilm reactors for bioremediation

There are different types of biofilm reactors and these are grouped according to the way they are operated (Singh *et al*., 2006). These include: biofilm fluidized bed (BFB), continuous stirred tank (CSTR - agitating continuous reactors, and rotary continuous reactors), trickling bed (TBR), airlift reactors (ALR), expanded granular sludge blanket (EGSB), upflow sludge blanket (USB) (Nicolella *et al*., 1999; Qureshi *et al*., 2005) and internal circulation (IC) (Nicolella *et al*., 1999) (Table 1.2).

In BFB, USB and EGSB, an upward liquid flow fluidizes the particles (Singh *et al*., 2006). CSTR can be defined as an ideally stirred tank reactor (ideally mixed reactor) which is equipped by at least a single impeller (Qureshi *et al*., 2005). In this system the rate at which the products are withdrawn and medium fed into the reactor is the same (Qureshi *et al*., 2005). It is characterized by a continuous flow of reactants and products and a homogenous mixture (Nelson & Sidhu, 2002). In CSTR, only fibrous bed support, which allows for vigorous movement can be used for adsorption of cells (Qureshi *et al*., 2005). This system has been used for butanol production from corn using *Clostridium acetobutylicum* cultures (Huang *et al*., 2004) but is now mostly used in studies involving combustion (Nelson & Sidhu, 2002).

USB reactors have been used for anaerobic treatment of wastewater/industrial effluents (Qureshi *et al*., 2005). This system is based on the development of dense granules (1–

4 mm) formed by the natural self-immobilization of the anaerobic bacteria. This kind of immobilization does not employ any support material such as Raschig rings or clay in the reactor (Nicolella *et al*., 1999). USB reactors have been used for anaerobic removal of pentachlorophenol (Ye, *et al*., 2004) N (Schmidt *et al*., 2004) and dechlorination using aerobic and anaerobic treatment of chlorophenol containing wastewaters (Shin *et al*., 1999).

Biofilm reactors offer several advantages over conventional suspended growth systems (Qureshi *et al*., 2005). A well known property of biofilm systems is their capacity to tolerate shock loads and to offer high cell concentrations (Zilouei *et al*., 2006). Carrier materials with adsorption or ion exchange properties allow for the buffering of high concentrations of toxic substrates which would otherwise exceed the tolerance of the bacteria (Panke *et al*., 1998).

Table 1.2 Some of bioreactors that are used for hydrocarbon bioremediation (Singh *et al*., 2006).

Pollutant	Reactor or experimental conditions	Organisms or culture	Overall efficiency of degradation
Chlorophenols			
2-Chlorophenol	Silicone tube membrane bioreactor	Anaerobic sludge from a swine wastewater treatment plant	>90%
4-Chlorophenol	Granular activated-carbon biofilm reactor	Bacterial consortium from rhizosphere of *Phragmites australis*	70–100%
2,4-Dichlorophenol	Rotating perforated tube biofilm reactor	*Pseudomonas putida*	~100%
2,4,6-Trichlorophenol	Fluidized bed biofilm reactor	*Pseudomonas* sp., *Rhodococcus* sp.	~100%
2,3,4,6-Tetrachlorophenol	Fluidized bed biofilm reactor	*Pseudomonas* sp., *Rhodococcus* sp.	~100%
Pentachlorophenol	Fluidized bed biofilm reactor	*Pseudomonas* sp., *Rhodococcus* sp.	~100%
Pyrene, phenanthrene	Biofilm grown directly on liquid medium	*Polaromonas* sp., *Sphingomonas* sp., *Alcaligenes* sp., *Caulobacter* and *Variovorax* sp.	~50% (pyrene); ~98% (phenanthrene)
o-Cresol, naphthalene, phenol, 1,2,3-trimethylbenzene	Biofilm grown in NAPLs	*Pseudomonas fluorescens*	Not determined
n-Alkanes	Rotating biological contactors	*Prototheca zopfii*	~65%
Carbon tetrachloride	Continuous flow fixed biofilm reactor	*Providencia stuartii, Pseudomonas cepacia*	~100%
Toluene	Hollow-fibre membrane biofilter reactor; continuously fed biodrum reactor	Secondary sludge from wastewater treatment plant; various aerobic and anaerobic bacteria	~84%; 65%
Azo dyes			
Acid Orange 10, 14	Laboratory-scale rotating drum biofilm reactor	*Methylosinus trichosporium*	~60%
Everzol Turquoise Blue G	Laboratory-scale activated sludge unit	*Coriolus versicolor*	~82%
Herbicides			
MCPP; 2,4-D	Granular activated-carbon biofilm reactor	Mixed culture of herbicide-degrading bacteria	MCPP (partial); 2,4-D (complete)

1.6 Fluidized bed biofilm reactors (FBBRs)

Biofilm bioreactors can be defined as the class of bioreactors in which the bacteria acting as the biocatalyst (is) are found in an anchored or attached form, either on the surface of an inert "carrier" or attached to one another in flocs (Saravanan & Sreekrishnan, 2006).

FBBRs are highly efficient (Sokół & Korpal, 2004) and as such their use in the treatment of various industrial wastewaters has been increasing in popularity (García Encina & Hidalgo, 2005). To achieve the fluidization of the biocarriers or biofilm particles, the fluid velocity through the bed should be sufficient (10–20 m/h) to induce the settled bed to expand (Nicolella *et al*., 1999) (Figure 7). FBBRs have been successfully used for the treatment phenolic containing wastewaters (Worden & Donaldson, 1987; Hirata & Hosaka, 1989; Lin & Weber, 1991), coke oven wastewater (Hirata *et al*., 1991) and nitrite-nitrogen wastewater (Cooper & Williams, 1990).

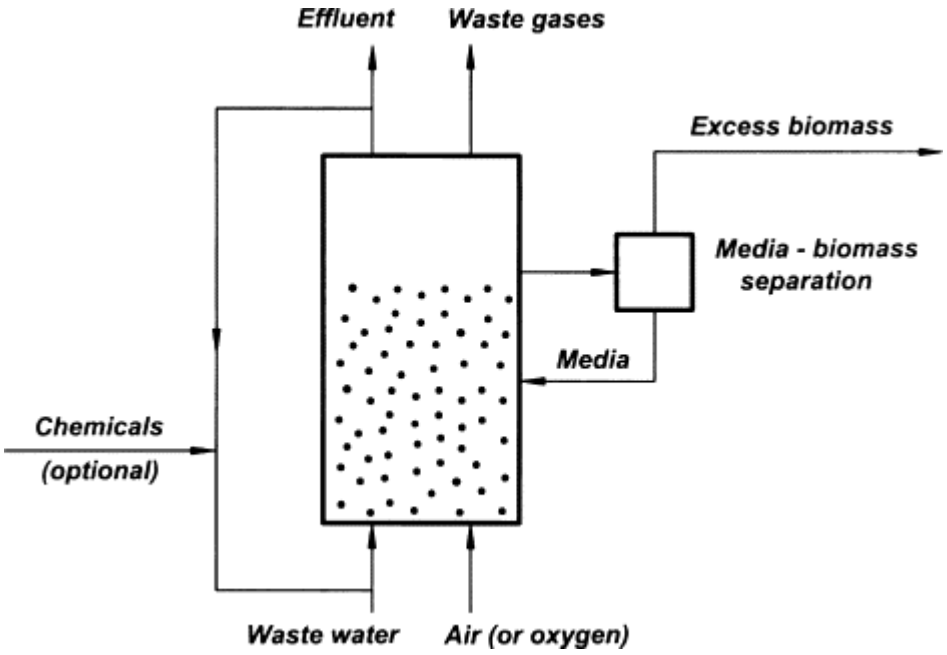

Figure 1.7 A simplified diagram of an aerobic fluidized bed bioreactor (Sokol & Korpal, 2006).

The technological success of the fluidized bed bioreactor is mainly due to the bioreactor's higher volumetric biomass concentration and surface area compared with the other suspended and attached growth dependent bioreactor systems (Lazarova & Manem, 1995; Bohlmann & Bohner, 2001). Other advantages that FBBRs have over the other high rate bioreactors, such as the USB, include higher bioremediation capacity, no clogging of the bioreactor bed, no problem of sludge washout and small volume and land requirements for setting up the bioreactor system (Heijnen *et al*., 1989).

In a study done by Rabah and Dahab in 2004 where the performance of the FBBR in the denitrification of high-nitrate wastewater was investigated, it was discovered that

the FBBR had removal efficiency of 99.8%. In that study, it was concluded that higher denitrification rates could be obtained at lower velocities because it was at these velocities that high biomass concentration was maintained (Rabah & Dahab, 2004).

1.7 General research focus of the project

The main objective of this study is to investigate the biodegradation of sodium benzoate by a *Pseudomonas* consortium in an aerobic fluidized biofilm bioreactor. Benzoate is an ideal chemical as it is an intermediate in the biodegradation of several aromatic hydrocarbon contaminants and is safe to work with as a model substrate that can provide information on aromatic hydrocarbon degradation.

This research relates the investigation of benzoate degradation under batch and continuous modes by four of the nine identified species - *Pseudomonas putida* BDS1 (Accession number EU096503), *Pseudomonas aeruginosa* BDS2 (Accession number EU096504), *Pseudomonas putida* GR1 (Accession number EU111745) and *Burkholderia cepacia* GR3FAR (Accession number EU103614) in a fluidized biofilm reactor with main emphasis on the relationship between bacterial biomass and the rate of degradation.

Motivation

The use of aerobic biofilm bioreactors in biodegradation has become one of the most successful ways of aromatic hydrocarbon bioremediation recently (Qureshi *et al*., 2005). However, the success of these reactors depends on many things including retention time, and the flow rate. The efficiency of oxygen transfer is a major constraint in aerobic bioreactors. Also aeration of a fluidized bed will create additional shear forces on the biofilm coated carrier particles. In this study the impact of aeration will be ascertained by monitoring aeration effects on bacterial attachment, biofilm growth and development. The development of biological reactors for the treatment of toxic and recalcitrant organic pollutants is a complex task. Firstly, microbial inoculation, acclimation and selection must be optimized to provide the best microflora possible. Secondly, innovative technologies must be developed to overcome the intrinsic low degradation rates of recalcitrant pollutants in order to allow short treatment times. Finally, since the pollutants involved are often toxic, it is important to use well-managed treatment system that limit potential process hazards to human and environmental health. For all these to be achieved, the relationship between bacteria biomass production, influent flow rates and biodegradation rates must be known.

Approach:

Application of aerobic fluidized - bed biofilm bioreactor technology for the treatment of wastewater containing benzoate.

Objective:

Investigation of biodegradation of sodium benzoate by a *Pseudomonas* consortium in an aerobic fluidized bed biofilm reactor.

Specific aims:

- Isolate and identify sodium benzoate degrading bacteria.
- Establish and monitor biofilm formation
- Once biofilm has formed, monitor the relationship between biofilm biomass, bioreactor flow rate and benzoate depletion.
- Biofilm SEM studies.

Chapter 2

Isolation and identification of novel benzoate-degrading bacterial populations

Abstract

Sodium benzoate-degrading bacteria (nine isolates) from diesel polluted soils were selected and identified. For isolation of the desired bacteria, five grams of soil was added to 50 ml of water and shaken at 175 rpm for 1 hr at 4°C and serially diluted and plated onto King's medium B (KB). To ensure that only bacteria with hydrocarbon degrading capacities were selected, glycerol, which is used as a carbon source in the King's media, was replaced by a mixture of hydrocarbons, such as toluene and sodium benzoate. DNA was then extracted from the resulting colonies on agar medium and PCR performed. The PCR products were sequenced and the obtained sequences compared to those of known organisms by BLAST. The BLAST results were then used to construct a phylogenetic tree. The isolates were identified as *Pseudomonas putida* BDS1 (Accession number EU096503), *Pseudomonas aeruginosa* BDS2 (Accession number EU096504), *Pseudomonas putida* GR1 (Accession number EU111745), *Burkholderia cepacia* GR3FAR (Accession number EU103614)**,** *Bacillus macroides* SBSY4 (Accession number EU096501) and *Bacillus simplex* MAR (Accession number EU111744). The isolated *Pseudomonas* species were also genetically similar to *P. putida* DSS2 (DQ304685) and *P. aeruginosa* CSICO (DQ304683) which have previously been used in biodegradation studies of sodium benzoate. The discovery that the isolates were closely related to well known hydrocarbon-degrading strains suggested that methods of isolation and identification used for benzoate-degrading bacteria were efficient.

Introduction

Benzoate is a cheap and non toxic aromatic (Alvarez *et al*., 1998) produced during biodegradation of several other aromatics, such as phenols (Egland & Harwood, 1999). Study of bioremediation of such simple and harmless aromatic compounds gives a basic understanding of degradation and therefore facilitates in studies of more recalcitrant aromatics (Ampe *et al*., 1998). The most important step in preparing for bioremediation studies is finding microorganisms that are able to degrade the hydrocarbon of interest (Van Hamme *et al*., 2004). Bacteria with the ability to degrade C5+ aromatic compounds are easily isolated from polluted soils (Shen *et al*., 1998). In general, soils hold up to about 10^9 microorganisms per gram (Cavaletti *et al*., 2006; Eichorst *et al*., 2007). For isolation of bacterial populations, serial dilution methods have shown to be efficient and are still very popular (Janssen *et al*., 2002).

In recent years there has been a move from the use of tubes and standard references such as *Bergey's Manual of Systematic Bacteriology* or the *Manual of Clinical Microbiology* (Clarridge III, 2004) for bacterial identification, to the use of automatic ID systems (Fontana *et al*., 2005). Phenotypic methods which include cellular fatty acid profiles, carbon source utilization systems and staining behaviour (Bosshard *et al*., 2003) have also been used. As an alternative to phenotypic methods, genotypic methods have been used instead (Tang *et al*., 1998). This is mainly due to inaccurate identification of bacteria based on phenotypic characteristics as compared to genotypic based identification methods (Clarridge III, 2004).

Genotypic methods are based on DNA or RNA extraction (Badiane Ndour *et al*., 2007). They allow for identification of bacteria using conserved sequences (Tang *et al*., 1998) i.e. stable parts of the genetic code (Clarridge III, 2004), such as 16S rRNA gene sequences (Kirschner *et al*., 1993). Therefore, molecular methods (DNA sequencing of the 16S rRNA) are still the best methods for identifying bacteria at the species level (Fontana *et al*., 2005). The 16S rRNA can be compared among major branches of life, the *Archaea Bacteria* (prokaryotes) and *Eukarya* (Figure 2.1) (Clarridge III, 2004).

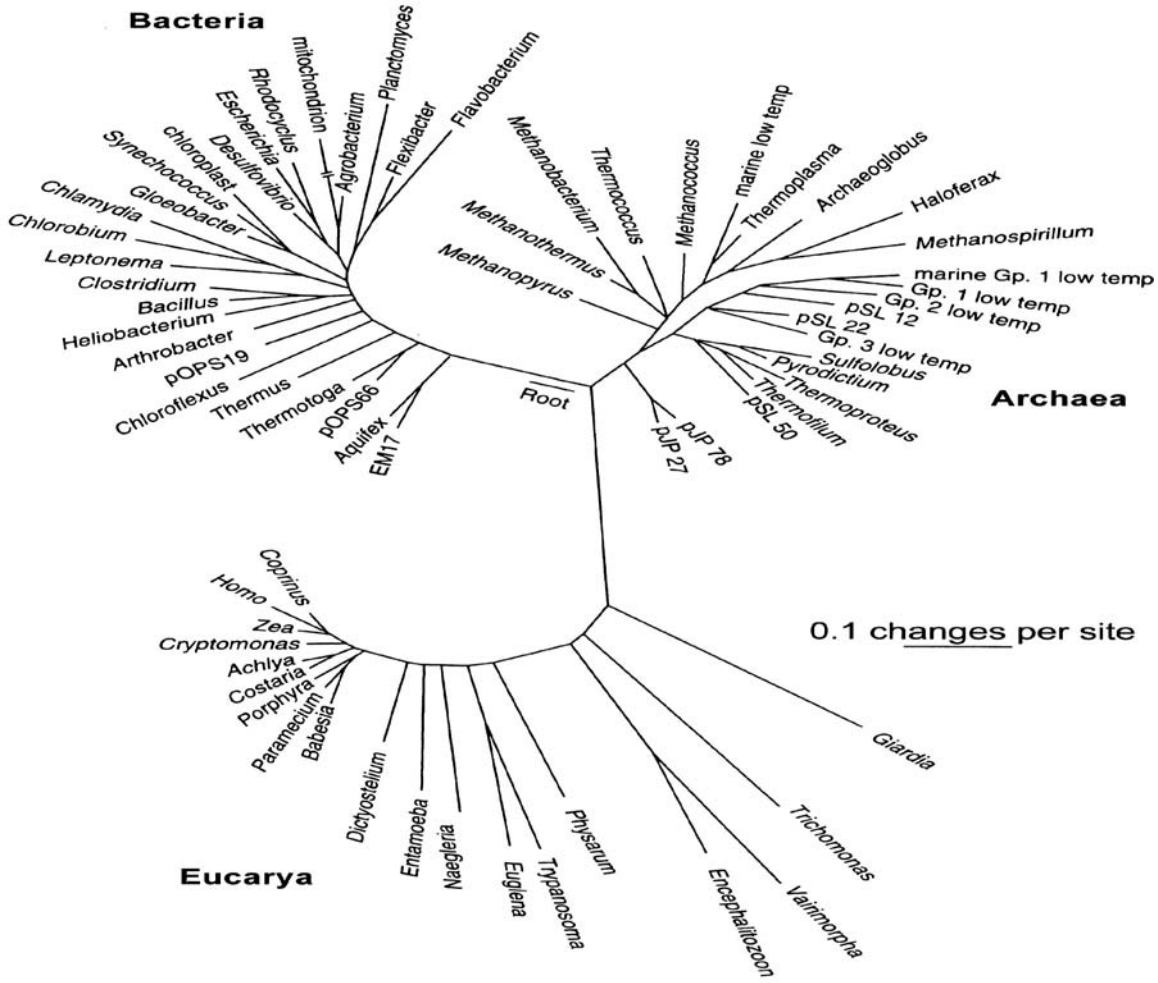

Figure 2.1 Universal phylogenetic tree based on the 16S rRNA gene sequence comparisons (Clarridge III, 2004).

The main goal of this study was to select isolates that were capable of degrading benzoate and to identify them by comparative analysis of partial 16S rRNA gene sequences.

Materials and Methods

1) **Bacterial strains**

Soils contaminated with various organic pollutants (e.g. soil exposed to continual diesel fuel spillage) were collected from industrial sites. To isolate bacteria from the soil samples, five grams of each soil sample was added to 50 ml of sterile water shaken at 175 rpm for 1 hr at 4°C. The resultant soil slurry was serially diluted and plated onto King's medium B (KB) with a mixture of hydrocarbons [e.g. 0.2% (v/v) toluene, 0.2% butanol (v/v) and 0.5% sodium benzoate (v/v) instead of glycerol. Plates were incubated for 3 days at 28°C. Nine resultant colonies were selected based on differences in colony morphology. The isolates were stored on Tryptone Soya Agar (TSA) (Oxoid) plates at 4°C (Lindsay *et al*., in press).

2) **Bacterial identification**

i) DNA extraction

DNA was extracted from each isolate using a modified boiling method described by Scarpellini *et al*., (2004). One colony of each isolate from stock TSA plates was boiled for 20 minutes in 40:l sterile distilled and filtered water plus 20:l chloroform, followed

by centrifugation at 12 000 rpm for 5 minutes. The supernatant was used as the DNA template during PCR reactions (Lindsay *et al*., in press).

ii) PCR and 16S rDNA sequencing

PCR and 16S rRNA sequencing were carried out as previously described by Lindsay *et al*., (in press). The primer set used for the amplification of 16S rDNA was U1392R (5'-ACG GGCGGT GTG TRC-3'; Lane *et al*. 1991; Ferris *et al*. 1996; McGarvey *et al*. 2004) and Bac27F (5'-AGA GTT TGA TCM TGG CTC AG-3'; Inagaki *et al*. 2003; McGarvey, *et al*. 2004) in combination with 2X PCR Master Mix (Fermentas Life Sciences, www.fermentas.com), according to the manufacturers instructions, and yielding a product of approximately 1300 bp. PCR amplifications were performed using the following conditions: initial denaturation of template DNA at 94°C for 3 min; then 35 cycles consisting of denaturation (94°C, 30s), annealing (60°C, 45s), extension (1min 30s, 72°C), and a final extension at 72°C for 7 min. The purified PCR products were sequenced and the resulting sequences analyzed by BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) against 16S rDNA sequences from GenBank (GenBank database of the National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/GenBank/) after confirmation of consensus sequences. A phylogenetic tree highlighting the clustering of the isolates was constructed using the neighbour joining method and bootstrapping in DNAMAN version 4 (Lynnon Biosoft) (Lindsay *et al*., in press).

Results and discussion

The results revealed that seven of the nine isolates clustered within the *Pseudomonas* genus (Figure 2.2), more specifically isolates BDS1 (Accession number EU096503), GR1 (Accession number EU111745) GR3FAR (Accession number EU103614) and FAR1 (Accession number EU103616) clustered within the *P. putida* group. These isolates were genetically similar (99%) to *P. putida* DSS2 (DQ304685) and *P. putida* strain BM2 (DQ989291). The former has previously been associated with sodium benzoate degradation (Lindsay *et al*., in press) (Figure 2.2).

BDS1 strain (EU096503), was found to be 100% genetically similar to *P. putida* strain RW-20 (DQ141543), isolated from arsenic-contaminated abandoned mine areas (Chang & Kim, 2005). Isolate BDS2 (EU096504) clustered within the *P. aeruginosa* group and was genetically similar (99%) to *P. aeruginosa* CSICO (DQ304683), a previously isolated sodium benzoate degrading *Pseudomonas* strain (Lindsay *et al*., in press). GR3FAR strain (EU103614) had a 99% genetic similarity to *B. cepacia* strain ATCC 53795 (AY741354) which was originally isolated from the rhizosphere of peas grown in soil from (King & Parke, 1996) and SBSY5 isolate (EU096502) was 99% genetically similar to *P. stutzeri* isolate C4 (AY880206) isolated from hides.

Interestingly, two *Bacillus* species, *Bacillus macroides* SBSY4 (EU096501) and *Bacillus simplex* MAR (EU111744), were also isolated in this study. It was not surprising, though, that some isolates identified belonged to that genus, as many species

of *Bacillus* have been reported to degrade a variety of recalcitrant aromatics such as 2,4,6-trinitrotoluene (TNT) (Kalafut *et al*., 1998). The two isolates both clustered within the *B. macroides* and *B. simplex* groups (Figure 2.2).

The isolates *B. macroides* SBSY4 and *B. simplex were* 99% genetically similar to *B. simplex* strain WN570 isolated from granite (Fajardo-Cavazos & Nicholson, 2005) and *B. macroides* (AF157696) isolated from the midgut of the Colorado potato beetle. Some soil isolates used that have been used in degradation wild bird feathers have been reported to be genetically similar to *B. macroides* (AF157696). On the other hand, *B. simplex MAR* (Accession number EU111744) was found to be 100% genetically similar to *B. simplex* strain WN579 (DQ275178), also previously isolated from granite (Fajardo-Cavazos & Nicholson, 2006).

As mentioned earlier, all the bacterial strains used in this study were isolated from soils contaminated with various organic pollutants. This would mean that in response to the presence of these compounds in their habitats, the isolates may have acquired new genetic information (Leahy & Colwell, 1990). It is believed that the new genetic material gives the bacteria the ability to utilize or transform the compounds for growth (Ma *et al*., 2006). Bacteria can gain genes from their closely related or even distant relatives by Horizontal Gene Transfer (HGT) (De la Cruz & Davies, 2000).

To select for hydrocarbon degrading bacteria, selective King's medium B (KB) with a mixture of hydrocarbons [e.g. 0.2% (v/v) toluene, 0.2% butanol (v/v), 0.5% n-

hexadecane (v/v) and 0.5% commercial motor oil (v/v)] as sole carbon sources was used. This means that only Pseudomonads with the ability to use those hydrocarbons would grow on that media. This would explain why the identified isolates were closely related to each other and to species reported to have hydrocarbon degrading capacities.

Both *P. aeruginosa* and *P. putida* have been linked with aerobic degradation of aromatics and have been reported to be amongst the most important hydrocarbon degrading bacteria (Leahy & Colwell, 1990). Therefore, the dominance of strains closely related to these two species would imply that the isolate could be used in hydrocarbon degradation studies.

Conclusion

In this study, potential hydrocarbon-degrading bacterial strains were successfully isolated from soil. The isolates were identified as several species including *P. putida*, *P. aeruginosa*, *P. stutzeri*, *B. cepacia*, *B. macroides* and *B. simplex*. These strains have the potential to be further used in biodegradation studies as they are genetically similar to previous biodegrading bacteria.

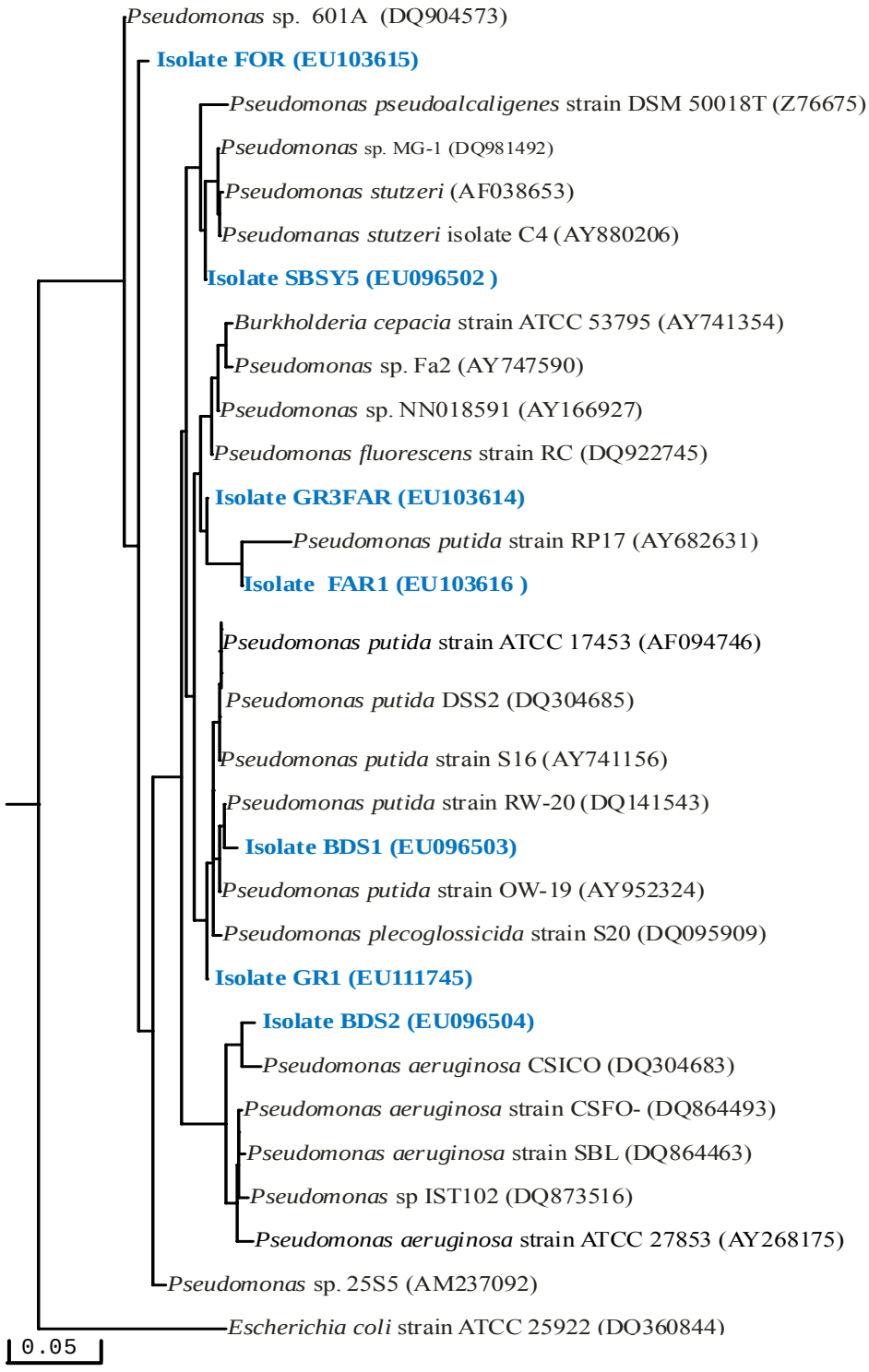

Figure 2.2 A phylogenetic tree rooted by outgroup (*E .coli*) and constructed using the neighbour joining method and bootstrapping in DNAMAN version 4 (Lynnon Biosoft). This was based on partial 16S rDNA sequences. Strains used were taken from BLAST results on NCBI.

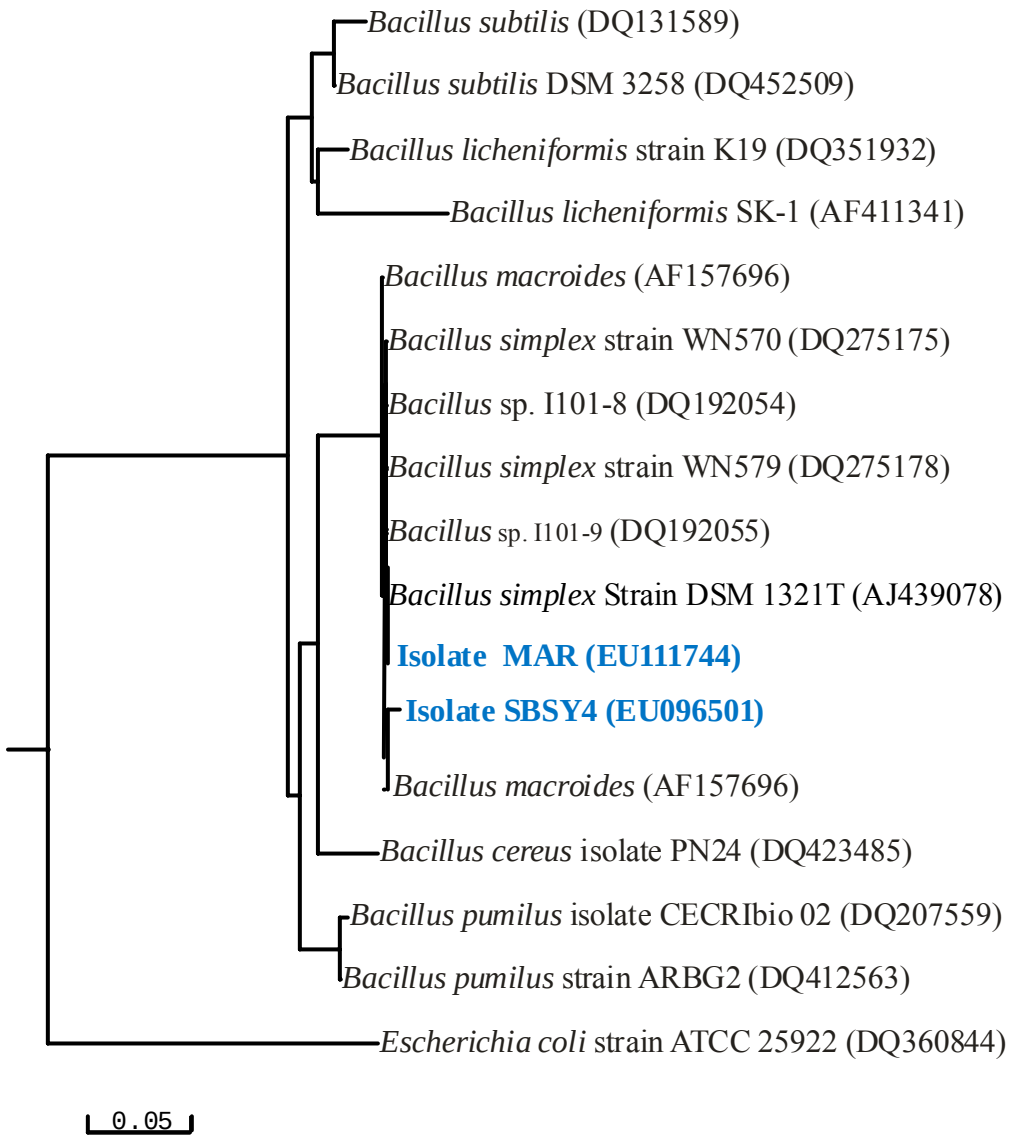

Figure 2.3 A phylogenetic tree rooted by outgroup (*E .coli*) and constructed using the neighbour joining method and bootstrapping in DNAMAN version 4 (Lynnon Biosoft). This was based on partial 16S rDNA sequences. Strains used were taken from BLAST results on NCBI.

CHAPTER 3

Biodegradation of sodium benzoate in a fluidized bed biofilm reactor (FBBR)

Abstract

Fluidized bed biofilm rectors (FBBRs) have been successfully used for decades in wastewater treatment. In this study, laboratory-scale FBBRs were used to degrade sodium benzoate. Sodium benzoate was a hydrocarbon of choice because while harmless and simple in structure, its study would aid in elucidating biodegradation of more complex toxic aromatics. Duplicate FBBRs containing 100g granular activated carbon (GAC) as carrier particles, were seeded with either a mixed inoculum of *P. aeruginosa* (BDS2), *P. putida* (BDS1 and GR1) and *B. cepecia* (GR3FAR), or a mixed inoculum including the four mentioned *Pseudomonas* species combined with two *Bacillus* species –*B. macroides* (SBSY4) and *B. simplex* (MAR). Controls experiments included FBBRs without GAC or without the bacterial inoculum or containing only the two *Bacillus* species. Reactors were operated under batch conditions, and continuous conditions at various flow rates including; 25ml/min, 85ml/min, 100ml/min, 140ml/min and 170ml/min. Scanning electron microscopy studies were also performed to observe morphology of attached populations on GAC. All biofilm reactors maintained high biomass concentrations compared to freely suspended cells, these reactors also showed higher degradations compared to their corresponding suspended cells. This could be explained by findings that immobilized cultures are associated with high density of active cells and good operational stability which results in improved reaction rates. Compared to continuous systems, batch systems showed more biodegradation and would therefore be a method of choice in bioremediation of hydrocarbons. SEM micrographs indicated that the strains were capable of forming biofilms. Most cells

from experiment reactors were abnormally long, suggesting nutrient depletion in those reactors.

Introduction

Bioreactors are vital in biochemical industries as their reaction rates have an impact on productivity (Maddox, 1989; Qureshi *et al*., 2005). For example, high reaction rates can be achieved by increasing cell concentrations in bioreactors (Qureshi *et al*., 2005). Membrane reactors (Mehaia & Cheryan, 1984) and immobilized cell reactors (Maddox, 1989) have been reported to have high reaction rates since they are capable of maintaining high biomass densities (Mehaia & Cheryan, 1984; Qureshi *et al*., 2005). Other simple reactors involve adsorption of microbial cells to form layers of cells or biofilm bioreactors (Qureshi *et al*., 2005).

Examples of biofilm reactors are fluidized bed reactors (FBRs) and continuous stirred tank reactors (CSTR) (Singh *et al*., 2006) and several others (Figure 3.1). Biofilm reactors are known for their success in wastewater treatment and they owe their success to high reaction rates compared to other types of reactors, which results from high cell concentrations achieved by biofilms in these reactors (Qureshi *et al*., 2005; Zilouei *et al*., 2006). Of all these types, FBRs have been very successful in remediation of toxic aromatics (Qureshi *et al*., 2005)

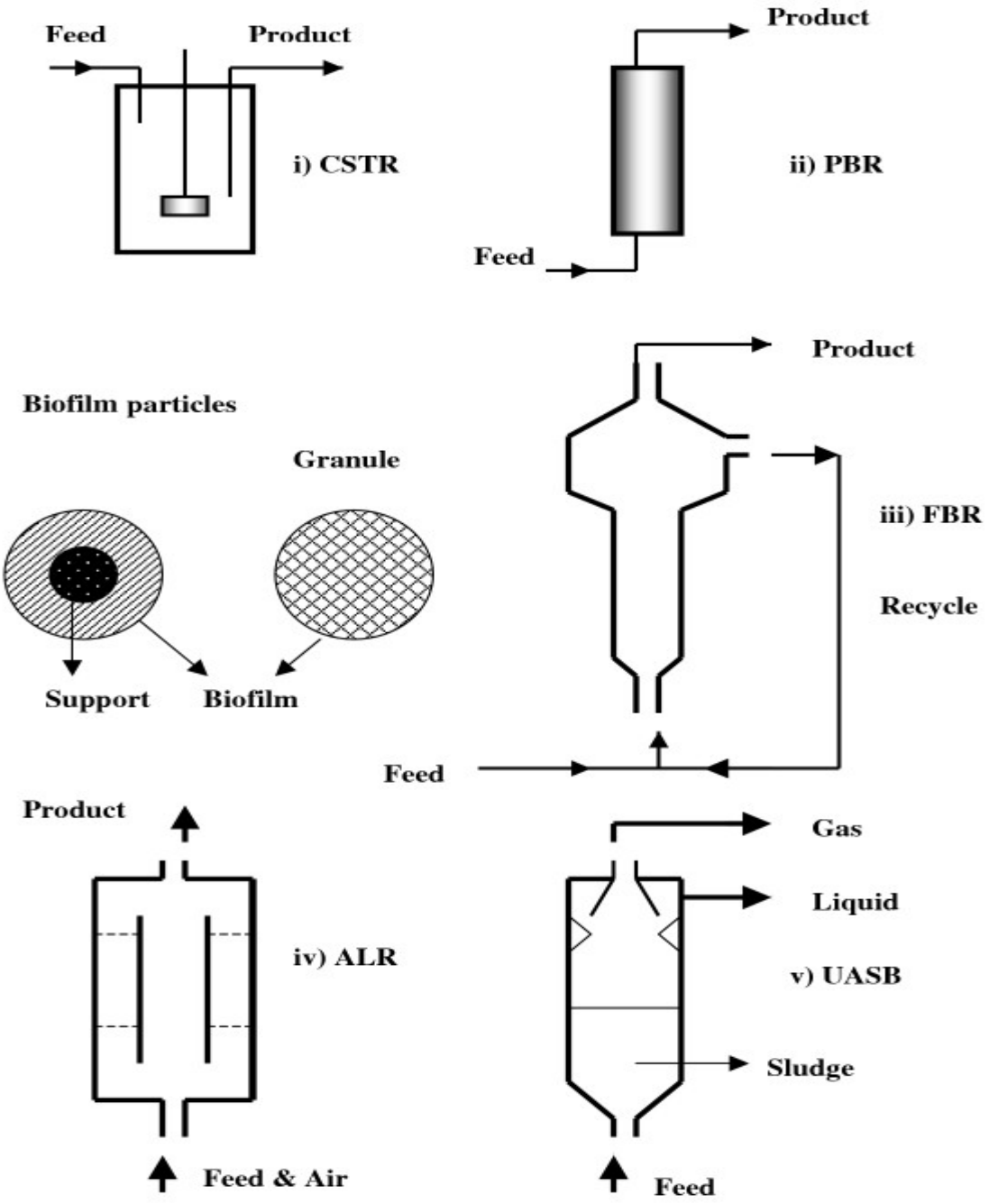

Figure 3.1 Diagrams showing different types of biofilm reactors i) continuous stirred tank reactors (CSTR), ii) packed bed reactor (PBR), iii) fluidized bed reactor (FBR), iv) airlift reactor (ALR) and v) upflow anaerobic sludge blanket (UASB) (Qureshi *et al*., 2005).

FBRs, also called fluidized bed biofilm reactors (FBBRs) (Qureshi *et al*., 2005), consist of biofilms adhered to inert moving support particles (Souza *et al*., 2004) and are therefore classified as particle-based reactors (Zilouei *et al*., 2006). They emerged for the first time in wastewater treatment in 1930s (Souza *et al*., 2004) but were only used in industrial wastewater treatment in the 80s (Souza *et al*., 2004; Qureshi *et al*., 2005).

Due to their high performance (Tang *et al*., 1987; He & Ping, 1994; Qureshi *et al*., 2005) FBBRs have been of great interest (Tang *et al*., 1987; He & Ping, 1994). In addition to the mentioned advantages, FBBRs offer many other advantages compared to other reactors (Zilouei *et al*., 2006). For example, minimal problems of diffusion, gas holdup and sludge retention, the liquid phase is evenly distributed throughout the reactor and most importantly high specific surface area offered to microorganisms (Bohlmann & Bohner, 2001; Ochieng *et al*., 2003) resulting in elevated microbial activity and high efficiency (Saravanane & Murthy, 2000).

In this study, mixed species biofilms, including Gram-Negative and Gram-Positive bacterial strains, were evaluated within different reactors, in either batch or continuous configurations.

Materials and Methods

1) **Bioreactor Description**

The FBBR used in this study was constructed from clear perspex (acrylic) (Figure 3.2). The column was 46cm high and could hold a volume of 3L. The internal diameter where granular activated carbon (GAC) (Associated chemical enterprises, Southdale, RSA) was fluidized was 10cm. The reactor had a water jacket for temperature regulation and was operated at 30º C. For aeration, air was supplied to the bed through cylindrical PVC holes that surrounded the bottom of the column. The holes had a diameter of 5mm each. Masterflex® and Tygon® tubing was used to connect the reactor to the air pump, water bath peristaltic pump, and the medium reservoir. The medium was fed in, at the bottom of the column (Figure 3.2).

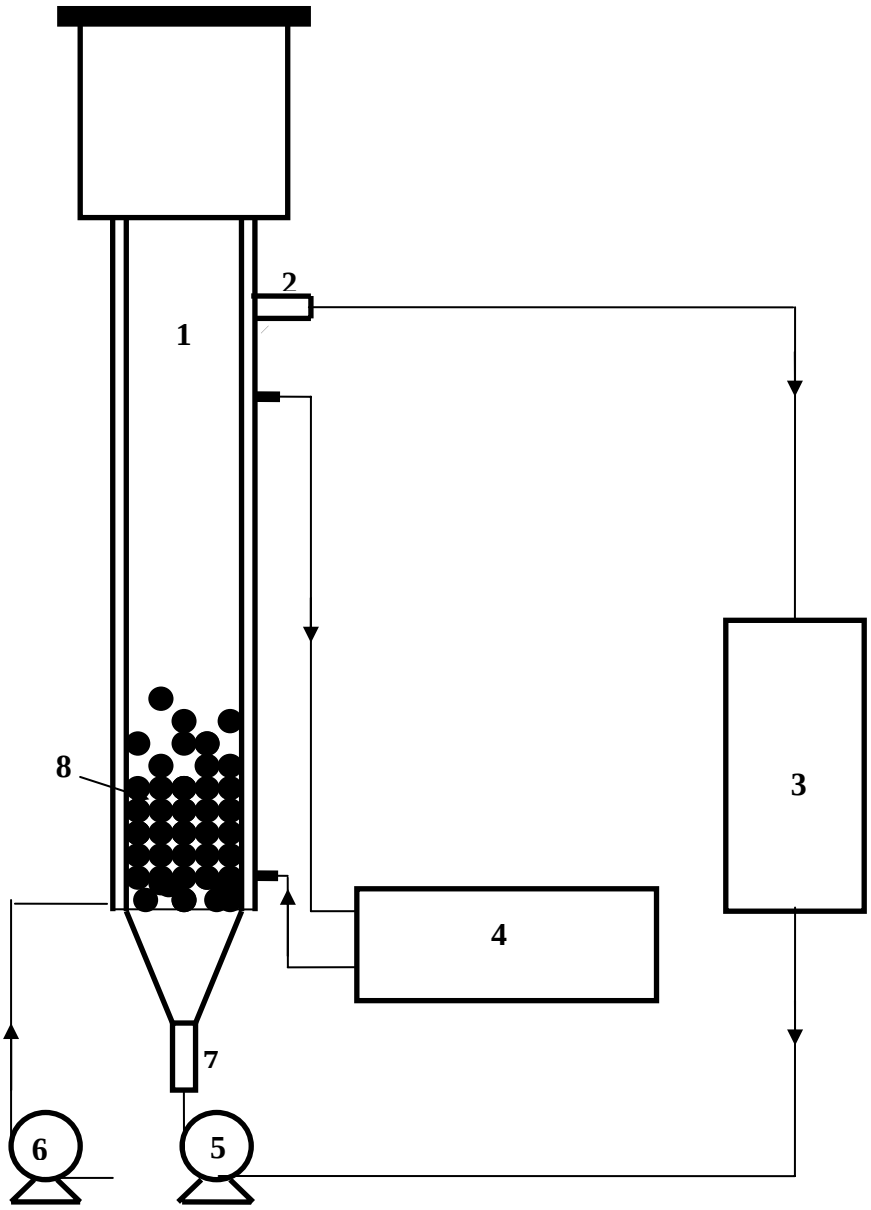

Figure 3.2 Schematic illustration of FBBR used. 1- Column, 2- FBBR outlet, 3 reservoir, 4-waterbath (30ºC) 5 – peristaltic pump, 6- Air pump, 7- FBBR inlet, 8- GAC.

2) **Sterilization of FBBR**

Prior to inoculation, the bioreactor was rinsed with 20% sodium hypo chlorite-containing sanitizer for 1 hour followed by neutralizing buffer (Difco) for 1 hour and then flushed with sterile distilled water as previously described (Lindsay *et al*., 2002).

3) **Bacterial isolates**

Four *Pseudomonas* strains identified as *P. putida* BDS1 (Accession number EU096503), *P. aeruginosa* BDS2 (Accession number EU096504), *P. putida* GR1 (Accession number EU111745) and *B. cepacia* GR3FAR (Accession number EU103614) were used together with two *Bacillus* strains – *B. macroides* SBSY4 (Accession number EU096501) and *B. simplex* MAR (Accession number EU111744) (chapter 2).

4) **Growth curves of isolates**

Growth curves of the isolates were generated by standardizing overnight cultures grown in 50 ml nutrient broth (NB) (Biolab, Wadeville, Gauteng, RSA). The overnight inoculum was diluted 1/100 from which 0.5 ml sample was then transferred into 50ml of NB and grown in an automated shaking incubator at 30^0C. Duplicate serial dilutions (up to 10^{-7}) were carried out every 4 hrs over a 24-h period interval and the number of

colonies (log cfu/ml) was determined by incubating the plates at 30^0C following plating by standard spread methods on nutrient agar (NA) (Biolab, Wadeville, Gauteng, RSA) (Baker *et al*., 1983). The experiment was done in two replicates. The resulting CFU values were used to construct growth curves. The growth curves were used to determine the species optimum growth as that would mean optimum biofilm growth (Else *et al*., 2003).

5) **Inoculum preparation**

Each isolate was grown in 50ml NB for 8hrs at 30^oC (mid-log growth phase for most isolates). Then 5ml of each isolate was used to inoculate the growth medium circulating in the bioreactor as described below.

6) **Biofilm establishment**

GAC was used as a biofilm carrier or attachment medium (100g) in each FBBR due to its slight electro-positive charge making it more attractive to chemicals and impurities. Different functional groups on the GAC also increase microbial attachment (Weber *et al*., 1979; Carvalho *et al*., 2001) while its rough surface provides excellent shelter and protection for bacterial attachment (Carvalho *et al*., 2001).

i) ***Pseudomonas*-based biofilm bioreactors (labeled A)**

Duplicate fluidized bed bioreactors, setup and sterilized as previously described, were inoculated with 1ml of a mid-log phase culture of each isolate of *P. putida* BDS1 (Accession number EU096503), *P. aeruginosa* BDS2 (Accession number EU096504), *P. putida* GR1 (Accession number EU111745) and *B. cepacia* GR3FAR (Accession number EU103614), grown for 8hrs in TSB at 30^{o}C. One – tenth NB (2L) was recycled through the reactor for three days to allow the biofilm to establish. The NB was then replaced by modified L9 growth medium plus 10g/l hydrocarbon and 5g/l sucrose (5ml to 2L medium). The L9 growth medium consisted of 3,6g/l of Na_2HPO_4, $2H_2O$, 3g/l of KH_2PO_4 , 0.5g/l of NaCl and 1g/l NH_4Cl and 2.5 ml of a trace element solution that consisted of 50 mg/l of $MnCl_2 \cdot 2H_2O$, 31 mg/l of H_3BO_3, 36 mg/l of $CoCl_2 \cdot 6H_2O$, 10 mg/l of $CuCl_2 \cdot 6H_2O$, 20 mg/l of $NiSO_4$, 30 mg/l of $Na_2MoO_4 \cdot 2H_2O$, and 50 mg/l $ZnCl_2$ and sodium benzoate (Bastiaens *et al*., 2000; Lindsay *et al*., in press). Since toxic aromatic hydrocarbons, such as toluene, are not safe to work with for our research purposes, sodium benzoate was used as a representative hydrocarbon-containing carbon source. Oxygen was supplied by directly aerating the column. The experiment was conducted on two separate occasions.

ii) **Mixed species-based biofilm bioreactors (labeled B)**

Duplicate FBBRs were prepared and inoculated with two *Bacillus* species - *Bacillus macroides* SBSY4 (Accession number EU096501)**,** *Bacillus simplex* MAR (Accession

number EU111744) and the four *Pseudomonas* species used in *Pseudomonas*-based biofilm bioreactors. Biofilms were allowed to develop in the reactors as described previously. The experiments were repeated on two separate occasions.

iii) **Control reactors**

Three types of control reactors were used during these experiments.

- To allow for comparisons of degradation between planktonic cells and biofilm cells (Sharanagouda & Karegoudar, 2002), control reactors were prepared without carrier particles and inoculated in the same manner with mixed species in order to monitor planktonic growth. Unlike the other controls, the reactors in this case were operated only under batch conditions and not continuous conditions. The experiment was conducted on two separate occasions. These reactors were labeled C1

- To check for the possibility of auto-degradation mechanisms such as photo-oxidation (Kot-Wasik *et al*., 2004) duplicate FBBRs were run without the bacterial inoculum and benzoate degradation monitored. These were operated under the batch mode followed by the continuous mode. The experiment was repeated on two separate occasions. The reactors were labeled C2

- To allow for comparisons of Gram-negative and Gram-positive (Fuller & Manning, 1997) based reactors, duplicate FBBRs were operated with two *Bacillus* species only. The support particles (GAC) were used as biofilm carriers. Biofilms were established as previously described and were initially operated under the batch system and later run under the continuous conditions (labeled C3). The experiment was conducted on two separate occasions

7) **Bioreactor Operation**

The bioreactor operation was performed in two phases; initial batch operation followed by continuous operation. Batch operation was performed mainly to produce biomass and to allow contact of the inoculum and GAC granules. This resulted in initial biofilm attachment on GAC particles. Then, the system was switched to continuous mode.

i) **Operation of bioreators as batch systems**

All the experiments in this study were initially operated under batch conditions. The medium (L9 Medium +10g/l hydrocarbon +5g/l sucrose) (5ml to 2L medium) was recycled for 20 days to allow for initial attachment of cells to GAC and fresh medium was supplied every 2 days. Sampling of liquid culture and GAC granules from the FBBR was done every second day and plate counts of attached and suspended cells were carried out as described in below. OD readings at 230 nm to monitor benzoate depletion were also measured every second day as described.

ii) **Operation of bioreactors as continuous systems**

After 20 days of operation under the batch mode, the operation of the reactors was switched to the continuous mode. The medium was continuously passed through the reactor at different flow rates (25ml/min, 85ml/min, 100ml/min, 140ml/min and 170ml/min). Planktonic counts, attached counts and OD readings were monitored over a period of 5 days at each of the flow rates.

8) **Enumeration of planktonic and attached populations**

Bulk fluid and GAC (5 ml of each) was sampled from the reactor every second day when the reactor was operated under batch conditions and every day when operated under the continuous mode. For attached bacterial measurements, GAC was rinsed with sterile distilled water, and then placed in 10 ml sterile 0.85% saline and 20g glass beads. These were then shaken vigorously by hand for 10 min (Lindsay *et al.*, 2002; Lindsay *et al*., in press). To allow injured cells to recover, the shaken solution was left to stand for 15 min. Serial dilutions in sterile 0.85% saline were prepared in duplicates and plated onto Nutrient Agar (NA) plates and Benzoate agar (BA) which consisted of 12g/l agar bacteriological plus 10g/l of sodium benzoate using standard drop plate method because it is faster and less laborious (Herigstad *et al*., 2001). Plates were

incubated at 30^0C overnight. The same dilution series and plating procedures were followed for the bioreactor fluid samples in order to enumerate planktonic populations.

9) **Monitoring benzoate biodegradation**

Bulk fluid (1ml) was sampled and centrifuged (12000 rpm) for 1 min. The supernatant was then removed by filtering into clean test tube and the OD readings taken. Benzoate absorbs best at 230 nm (Lindsay *et al*., in press) so to determine depletion, the OD readings were taken at that wavelength.

10) **Morphology of attached populations by SEM**

GAC granules (0.6g) were obtained from the reactor and rinsed with sterile distilled water. The granules were first fixed in 1ml of 3% aqueous glutaraldehyde at room temperature overnight. After fixation, they were rinsed with sterile distilled water once and dehydrated in graded ethanol series (20, 30, 40, 50, 60, 70, 80, 90. 95 and 100%) at 10 min intervals. They were then subjected to critical-point drying, mounted on SEM stubs and finally coated with normal gold/palladium for viewing on a Joel® 840 Scanning microscope (Lindsay *et al.* 2002). Cell sizes were also estimated on SEM images using the scale bar (Lindsay & von Holy, 1999).At least 20 cells were measured and averages calculated.

11) **Statistical analysis**

A) Attached and planktonic counts

Duplicates plate counts of attached and planktonic cells between replicates were converted to log cfu/ml, meaned, and standard deviations determined. The resulting counts were then compared to each other by one-way analysis of variance (ANOVA) using Stata 9/SE (Stata Corp., College Station, Texas, USA). The assumption of equal variances was checked for using Bartlett's test before ANOVA. If statistical significance was reached (P values <0.05 were considered statistically significant), then, post ANOVA data were separated using Bonferroni's multiple comparison test.

i) Batch system

Counts of attached and planktonic cells within reactors A, B and C3 were compared to each other as mentioned above. Attached cells obtained from reactors A and B were also compared to suspended cells in reactor C1 as described above.

ii) Continuous system

Counts in reactors A, B and C3 were compared at the rates 25ml/min, 85ml/min, 100ml/min, 140ml/min and 170ml/min as previously mentioned.

iii) Batch system vs Continuous system

Overall batch and continuous counts were compared by a two sample t-test (for two groups) assuming equal variances (Bartlett's test for equality of variances had been carried out prior to ANOVA; the t-test assumption was not violated). Stata 9/SE (Stata Corp., College Station, Texas, USA) at the 95% confidence level was used.

B) OD_{230} readings

i) Batch system

OD_{230} readings from reactors, A, B and C3 between replicates were meaned, standard deviation determined and the one-way ANOVA was used to determine significant differences ($P<0.05$) using Stata 9/SE (Stata Corp., College Station, Texas, USA). Bonferroni's multiple comparison test was used for post hoc comparison. Reactor A and B readings were also compared to C1 readings in the same manner.

ii) Continuous system

OD_{230} readings obtained from reactors A, B and C3 at different rates (25ml/min, 85ml/min, 100ml/min, 140ml/min and 170ml/min) were compared to each other in the as previously described.

iii) Batch vs Continuous

Overall OD_{230} readings obtained from reactors A, B and C3 were compared to each other as described above.

Results

Growth curves

In general, the isolates reached the mid-log phase after eight hours of incubation and showed maximum growth on the twelve hour, after which the stationary phase was reached. They maintained their stationary phase stage for about another twelve hours (Figure 3.3).

Control bioreactors

The goal of this study was to monitor counts of planktonic and attached cells in bioreactors and at the same time monitor sodium benzoate degradation under batch and continuous modes. In control reactors, the parameters monitored and the operation systems (batch and continuous) were limited by what the control reactors were lacking compared to the experiment reactors. For example, in bioreactors that were operated without GAC (C1), the reactors could not be operated under the continuous mode as all the cells in the bioreactor would be washed out, so only batch system results were reported, and only planktonic counts were monitored. Counts in these reactors reached

maximum of *ca* 4log cfu/ml after approximately 12 days of operation (Figure 3.4a). Degradation also occurred and the highest degradation was observed on day 20 (Figure 3.4b).

In control reactors where there was no inoculum (C2), the counts of both attached and planktonic cells were not monitored as there was no biomass in such reactors, therefore, only biodegradation was measured. In these reactors, biodegradation did not occur as OD_{230} measurements remained constant for the duration of experiments (Figure 3.4c), suggesting that degradation would be a result of microbial activity in the reactor.

In control reactors where *Bacillus* was used (C3), all the parameters (counts of planktonic and attached cells and benzoate degradation curves) were monitored under both batch culture and continuous culture conditions. In these reactors maximum counts of *ca* 5log cfu/ml were reached on the last day (day 20) (Figure 3.4d) of operation under batch operation. Degradation occurred in these reactors as there was a measurable decrease in OD_{230} readings with time (Figure 3.4f).

Experimental bioreactors

***Pseudomonas*-based biofilm bioreactors** (A) and **mixed species-based biofilm bioreactors (B) under batch operation.**

Initially, in reactors A and B, attached counts were *ca* 1 log and 2 log cfu/ml significantly (F = 105.64; df = 2, 117; P = <.0001) higher than their corresponding planktonic counts within the reactors on both benzoate agar and nutrient agar respectively (Figures 3.5a, b, d and e). From 16 days onwards, attached cells dominated

by *ca* 3log cfu/ml. With time, there was an increase in the adhered biomass and a decrease in planktonic cells (Figures 3.5a, b, d and e). However, attached counts obtained from reactor B were always *ca* 1log cfu/ml significantly (P = <.0001; 95% CI = 6.210 - 6.882; t = 5.561) higher than counts in reactor A (Figures 3.5a and e). OD_{230} measurements between reactor A and B on the other hand showed no significant differences (P = 0.156; 95% CI = 0.428 - 1.128; t = 0.5734), indicating the same degradation abilities in the two reactors. Compared to the control reactor containing only planktonic cells (C1), counts obtained from reactors A and B were significantly (F = 105.64; df = 2, 117; P = <.0001) higher by *ca* 2.4-3.5log cfu/ml and significantly (F = 26.93; df = 2, 117; P = <.0001) lower OD_{230} readings were recorded.

Compared to control reactors inoculated with two *Bacillus* species only (C3), there was significantly (F = 105.64; df = 2, 117; P = <.0001) more counts in reactors A and B. By the end of the experiments, the OD_{230} readings from reactors A, B and C3 were almost zero. However, the degree of reduction of the OD_{230} values varied depending on the type of inoculum used. In reactors A and C3 OD_{230} measurements were zero by day 18 (0.026) and day 20 (0.206), respectively (Figures 3.4f and 3.5c). By contrast, in reactors B, OD measurements of approximately zero were recorded after day 16 (0.003) (Figure 3.5f).

This study also showed that OD_{230} readings obtained from reactor A and B were significantly (F = 26.93; df = 2, 117; P = <.0001) lower than readings obtained from reactor C3 (Table 3.1). Thus, it appeared that degradation of sodium benzoate occurred faster in the presence of a mixed Gram-negative and Gram-positive consortium and in

the presence of Gram-negative inoculum alone, than in the presence of just Gram-positive consortium. This was demonstrated by the time it took for the OD_{230} values to be closest to zero. Decreased OD_{230} measurements implied decreased sodium benzoate concentrations in the bioreactor, and thus showed sodium benzoate as the sole carbon source utilized by the selected consortium of *Pseudomonas* and/or *Bacillus* species in this study.

Operation of reactors as continuous systems

In reactors A, B and C3, operated under these conditions, the counts of attached and planktonic cells increased with the increasing flow rates until the flow rate of 140ml/min. There was a noticeable decrease of plate counts at 170ml/min (Figures 3.6, 3.7 and 3.8 - a, b, d, e, g, h, j, k, m, and n). In all the three reactors, highest plate counts were obtained at 140ml/min. At this rate, highest attached counts of *ca* 6-8log cfu/ml were recorded from day 3, while highest planktonic counts of 4-6 log cfu/ml were achieved on day 1.

The results demonstrated that significantly (F = 32.20; df = 4, 295; P = <.0001), the number of cells increased with the increasing flow rates. Bonferroni's test, comparing means post ANOVA showed a significant difference at 140ml/min compared to rates 25ml/min (P< 0.0001) and 85ml/min (P = 0.08). However, a significant (F = 32.20; df = 4, 295; P = <.0001) decrease was observed at 170ml/min compared to all other rates (P = <.0001) (25ml/min, 85ml/min, 100ml/min and 140ml/min) in the three reactors.

This means that in general, cell counts increased in the following order: 25ml/min < 85ml/min < 100ml/min < 140ml/min.

The same trend was observed in biodegradation of sodium benzoate i.e. sodium benzoate depletion increased with the increasing flow rate when the reactor was operated at the highest rate of 140/ml. There was also a significant (F = 23.95; df = 4, 295; P = <.0001) decrease in degradation at 170ml/min shown by increased OD_{230} readings at that rate compared to OD_{230} readings obtained at 140ml/min (Figures 3.6, 3.7 and 3.8-c, f, i, l and o). Thus, there was a general increase of degradation rates with increasing flow rates.

Counts and degradation in batch and continuous systems

Comparatively, this study showed that, overall, degradation in batch systems were significantly (P = <.0001; 95% CI = 1.471 – 1.7688; t = -9.605) higher than in continuous cultures. However, there were significantly (P = 0.043; 95% CI = 5.490 – 5.885; t = -2.024) higher counts in continuous systems compared to batch systems.

The study also demonstrated that in reactors A, B, C1 C2, and C3 counts of cells grown in NA were significantly (P = <.0001; 95% CI = 4.049 – 4.339; t = 14.37) higher than their corresponding counterparts grown in BA indicating a preference for richer nutrient medium. Any growth on BA implied the ability of the cultured cells to utilize sodium benzoate as their sole carbon source**.**

Scanning electron microscopy (SEM)

SEM was used to observe biofilm morphology in fluidized bed biofilm reactors under batch system conditions. Scanning electron micrographs showed that bacterial cells were evenly distributed on the surfaces of GAC and preserved their rod shape features, as would be expected of both *Bacillus* and *Pseudomonas* species. Closer examination of GAC surfaces demonstrated bacterial cells embedded within a polymeric matrix (Figures 3.9a-b, d and 3.10a-d). Most cells exhibited smooth surfaces, while differing shapes and sizes were also seen (Figures 3.9 and 3.10). For example, biofilms associated with reactors A and B exhibited thinner and significantly (F = 28.50; df = 2, 27; P = <.0001) longer cells (3.515μm and 4.071μm respectively) (Figures 3.9 and 3.10), while shorter rods (0.994 μm) were observed from C3 reactors only (Figure 3.11) (Table 3.2).

Table 3.1 Highest and lowest counts and OD_{230} readings obtained from reactors C3, A and B, during batch and continuous modes of operation. * indicates a significant difference (P<0.0001)

		Counts (Log cfu/ml)			OD_{230} (Degradation of sodium benzoate)		
Reactor type	**Operation**	**Highest**	**Lowest**	**P value**	**Highest**	**Lowest**	**P value**
Bacillus only (C3)	Batch	4.880	3.350	<.0001*	3	0.206	<.0001*
	Continuous	6.771	3.249	<.0001*	3	1.411	<.0001*
Pseudomonas only (A)	Batch	7.355	4.388	<.0001*	3	0.001	<.0001*
	Continuous	8.395	4.341	<.0001*	3	0.821	<.0001*
Mixed species (B)	Batch	8.478	5.523	<.0001*	3	0	<.0001*
	Continuous	9.000	5.968	<.0001*	3	0.136	<.0001*

Table 3.2 Average lengths (n = 20) of *Bacillus*, *Pseudomonas* and mixed species cells from batch experiments as estimated from scanning electron micrographs. * indicates a significant difference (P<0.0001)

Bacteria	**Lengths (µm)**	**P value**
Bacillus	0.99	<.0001*
Pseudomonas	4.07	<.0001*
Pseudomonas and *Bacillus* (Mixed)	3.52	<.0001*

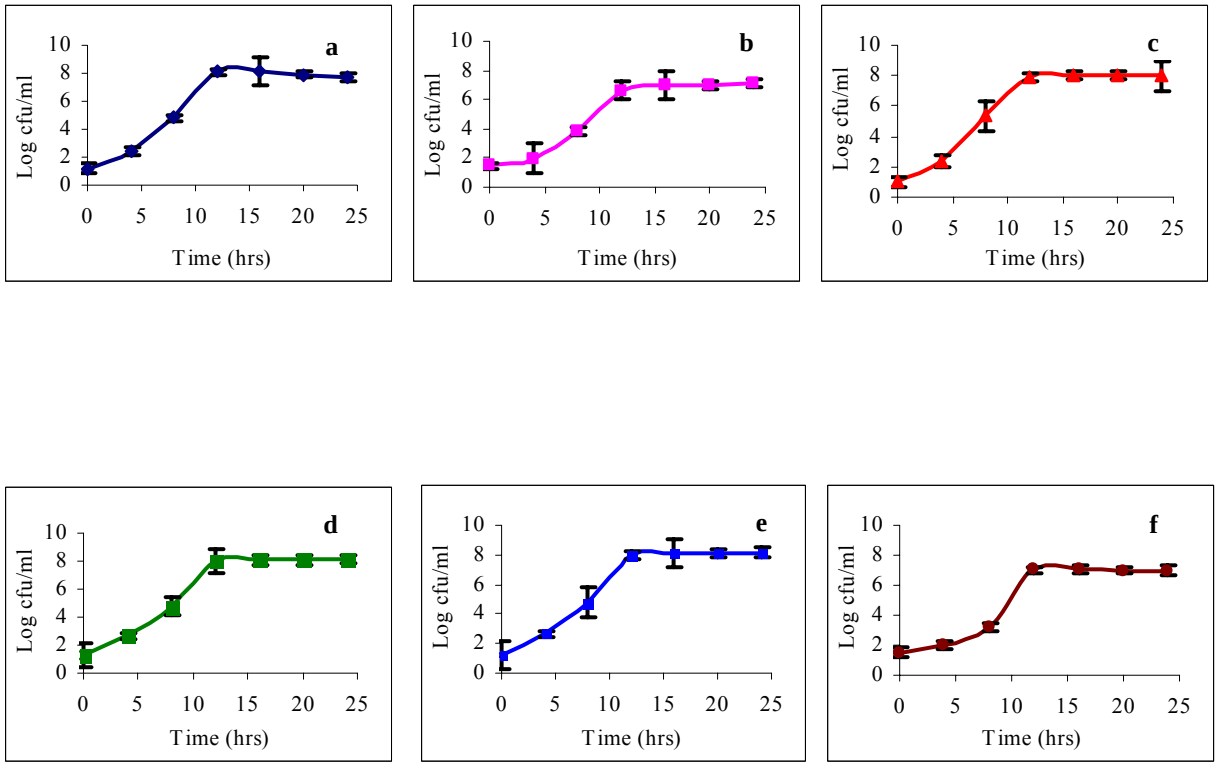

Figure 3.3 Growth curves of bacterial isolates BDS1 (a), BDS2 (b), GR1 (c), GR3FAR (d), SBSY5 (e) and MAR (f). The isolates were grown in nutrient broth and growth monitored every four hours over a 24 hr period.

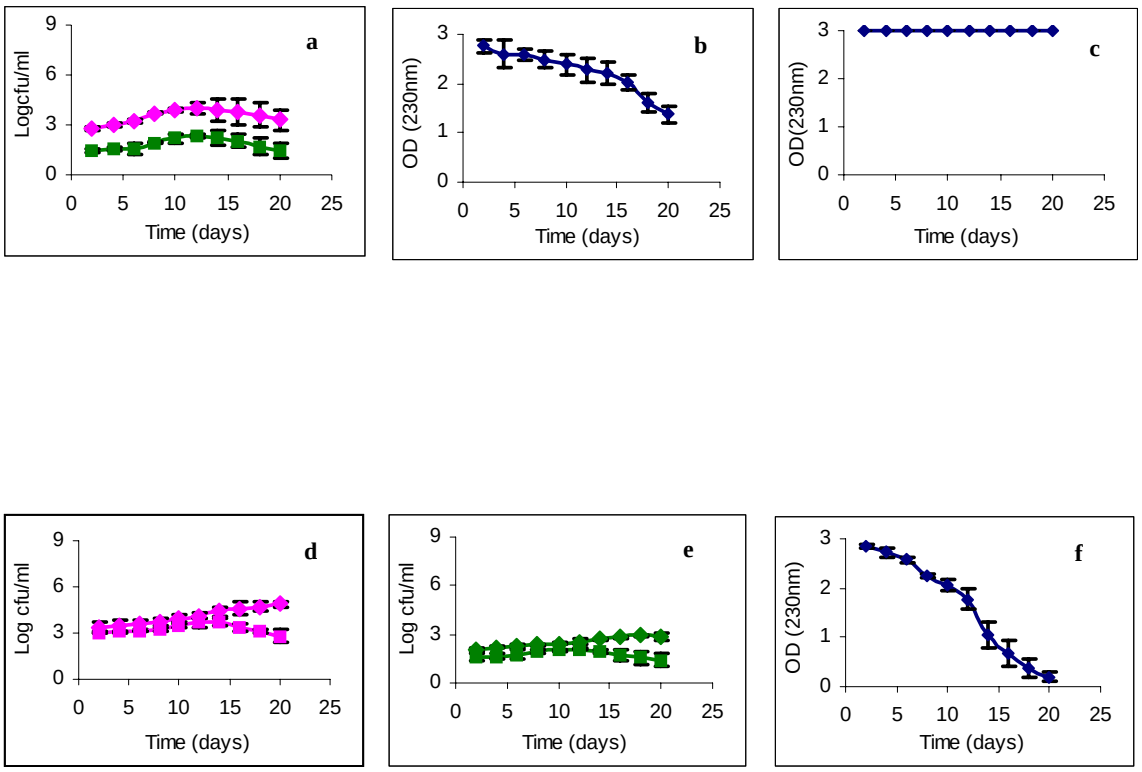

Figure 3.4 Counts (log cfu/ml) obtained from control bioreactors of suspended cells (C1) (a) and two *Bacillus* species (C3) (d) grown on NA (pink) and (e) BA (green) under batch mode of operation. In C3, counts of both attached (triangle) and planktonic (square) cells were recorded. Depletion curves of suspended cells (b), no inoculum (c) and two *Bacillus* species (f) are also shown.

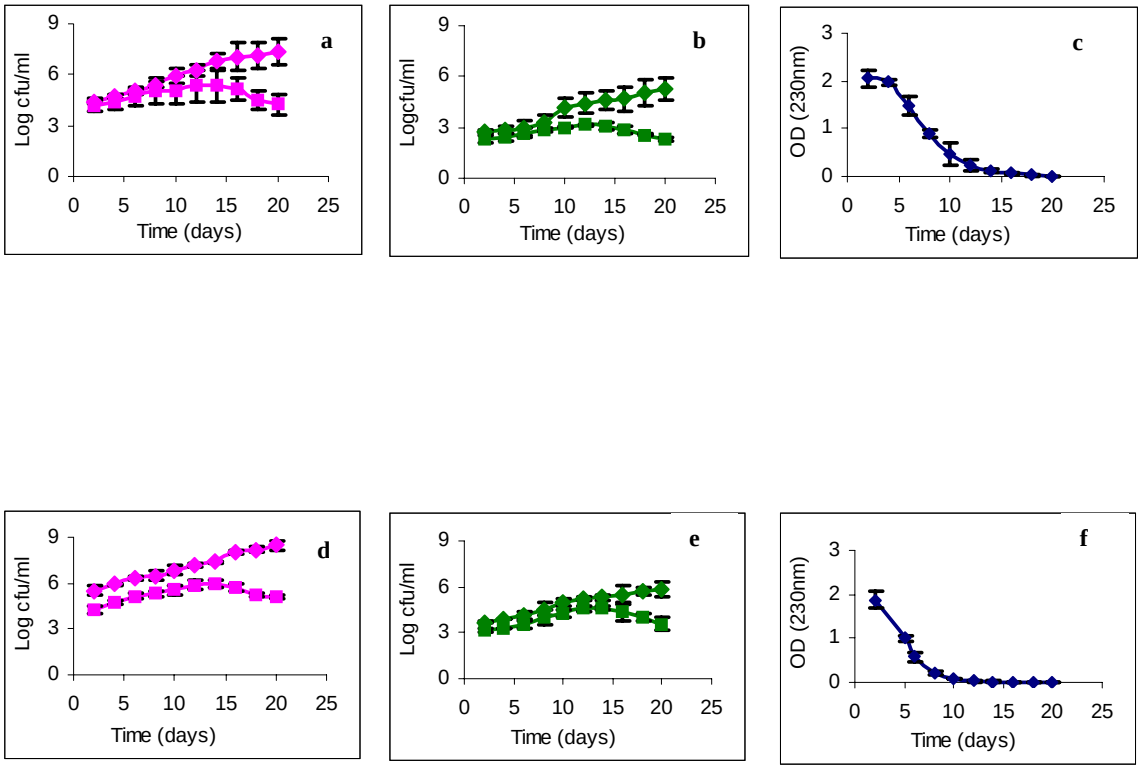

Figure 3.5 Batch culture counts (log cfu/ml) (a, b, d, and e) and depletion studies (c and f) of populations of *Pseudomonas* strains (a, b and c) and mixed cultures of *Pseudomonas* strains and *Bacillus* strains (d, e and f) attached (triangle) and planktonic (square) grown on NA (pink) and BA (green).

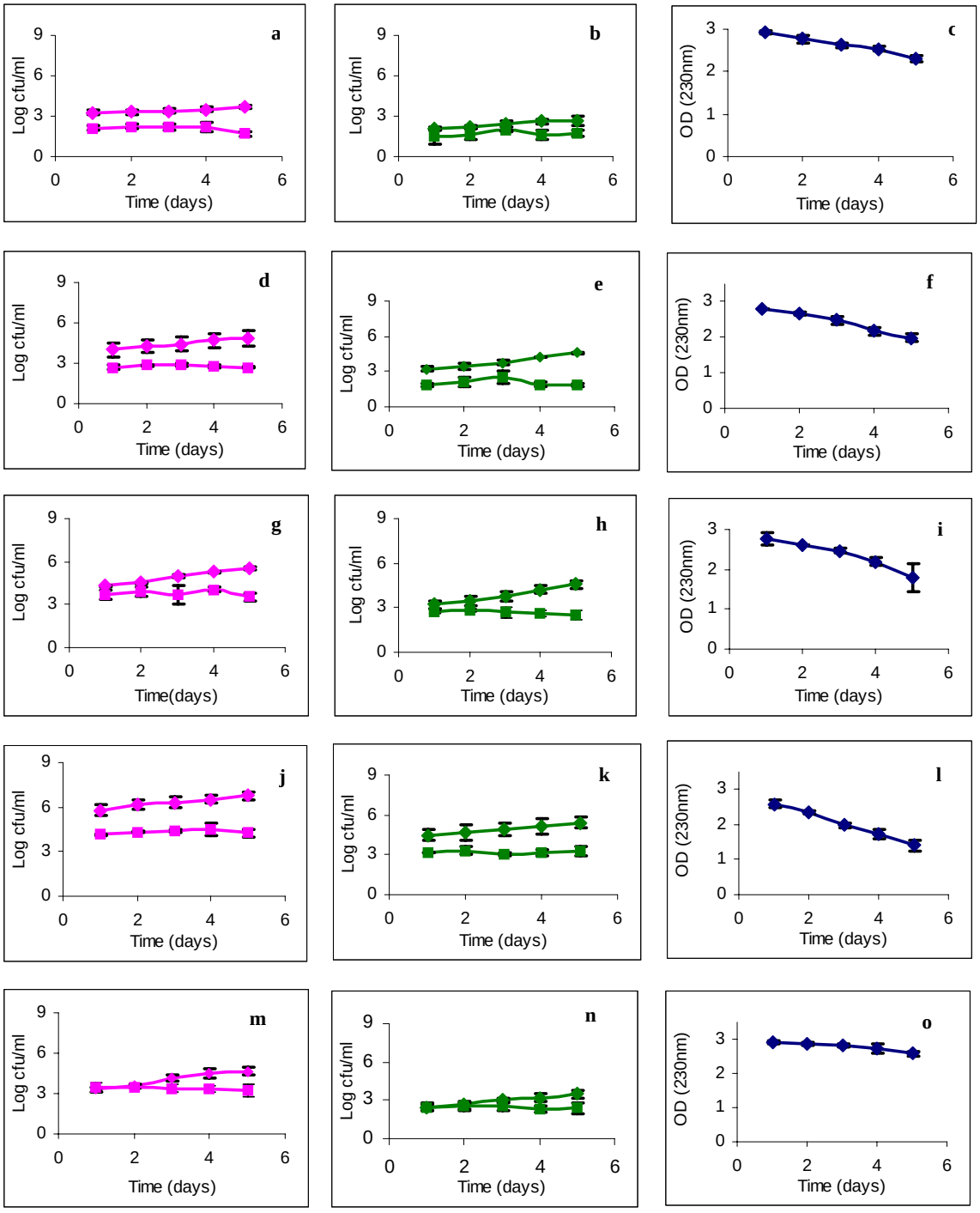

Figure 3.6 Counts (log cfu/ml) (a, b, d, e, g, h, j, k, m and n) and depletion studies (c, f, i, l and o) of *Bacillus* strains attached (triangle) and planktonic (square) grown on NA (pink) and BA (green) at flow rates of 25ml/min (a-c), 85ml/min (d-f), 100ml/min (g-i), 140ml/min (j-l) and 170ml/min (m-o).

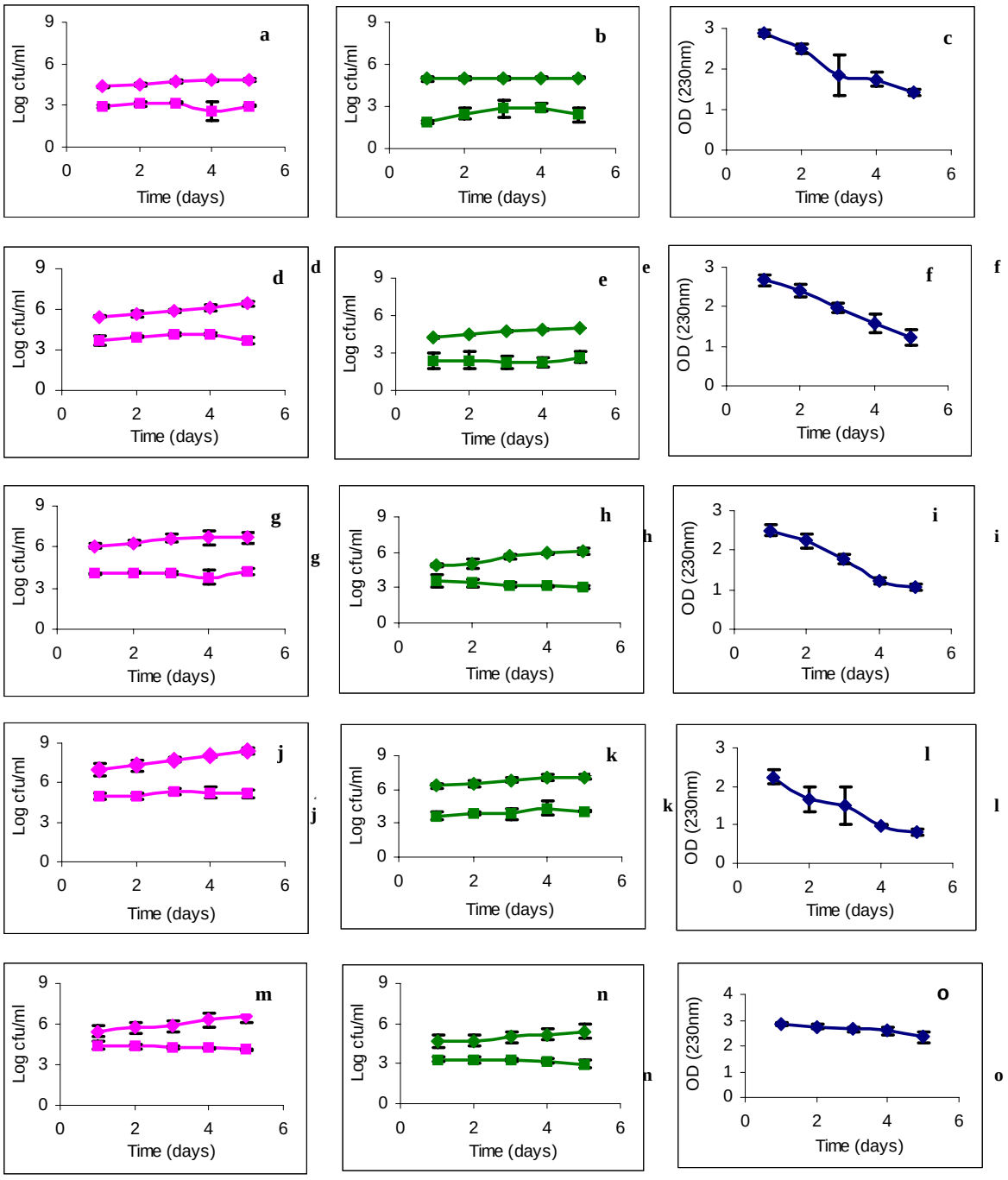

Figure 3.7 Counts (log cfu/ml) (a, b, d, e, g, h, j, k, m and n) and depletion studies (c, f, i, l and o) of populations of *Pseudomonas* strains attached (triangle) and planktonic (square) grown on NA (pink) and BA (green) at flow rates of 25ml/min (a-c), 85ml/min (d-f), 100ml/min (g-i), 140ml/min (j-l) and 170ml/min (m-o).

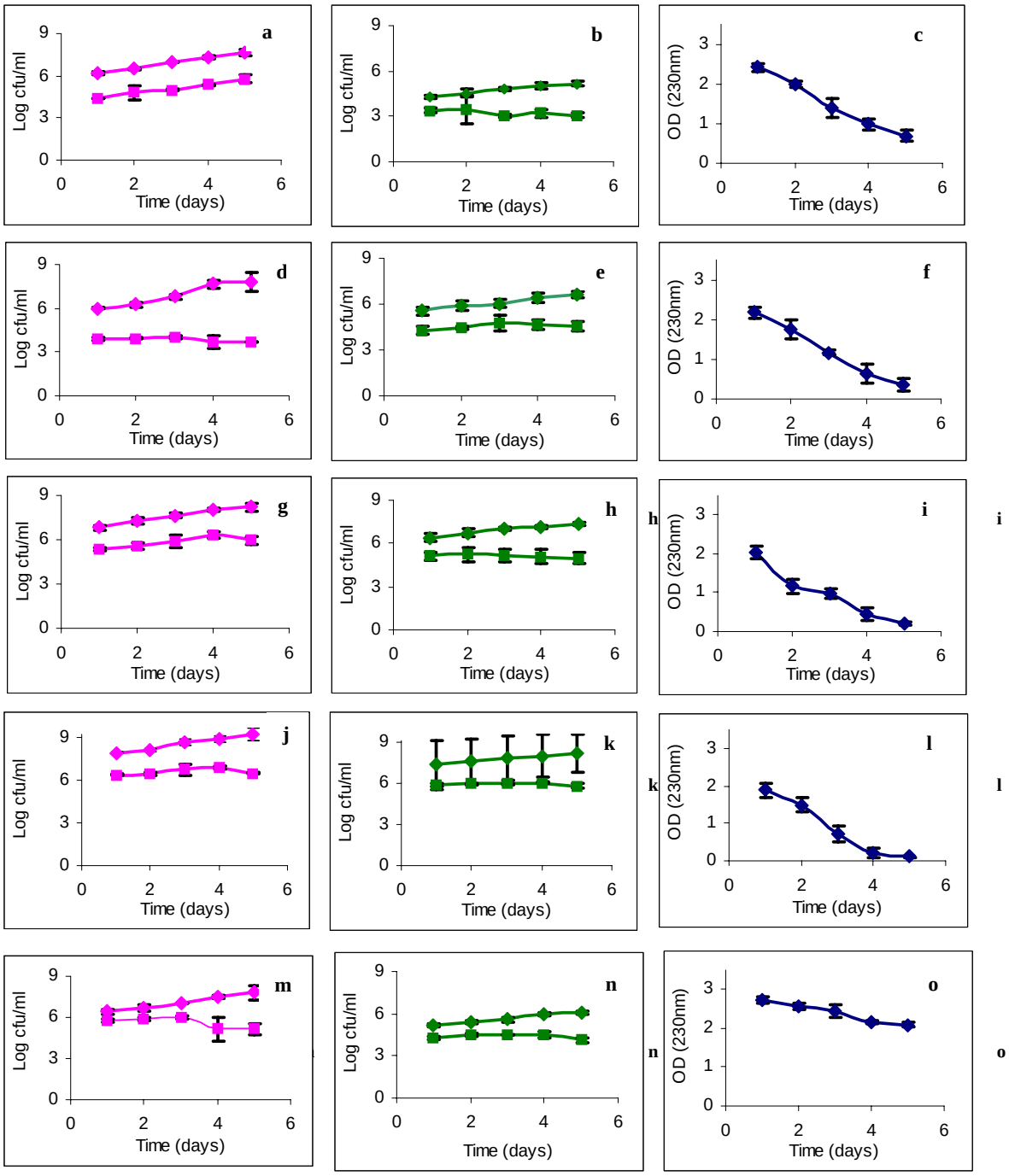

Figure 3.8 Counts (log cfu/ml) (a, b, d, e, g,h, j, k, m and n) and depletion studies (c, f, i, l and o) of mixed populations of *Pseudomonas* and *Bacillus* strains attached (triangle) and planktonic (square) grown on NA (pink) and BA (green) at flow rates of 25ml/min (a-c), 85ml/min (d-f), 100ml/min (g-i), 140ml/min (j-l) and 170ml/min (m-o).

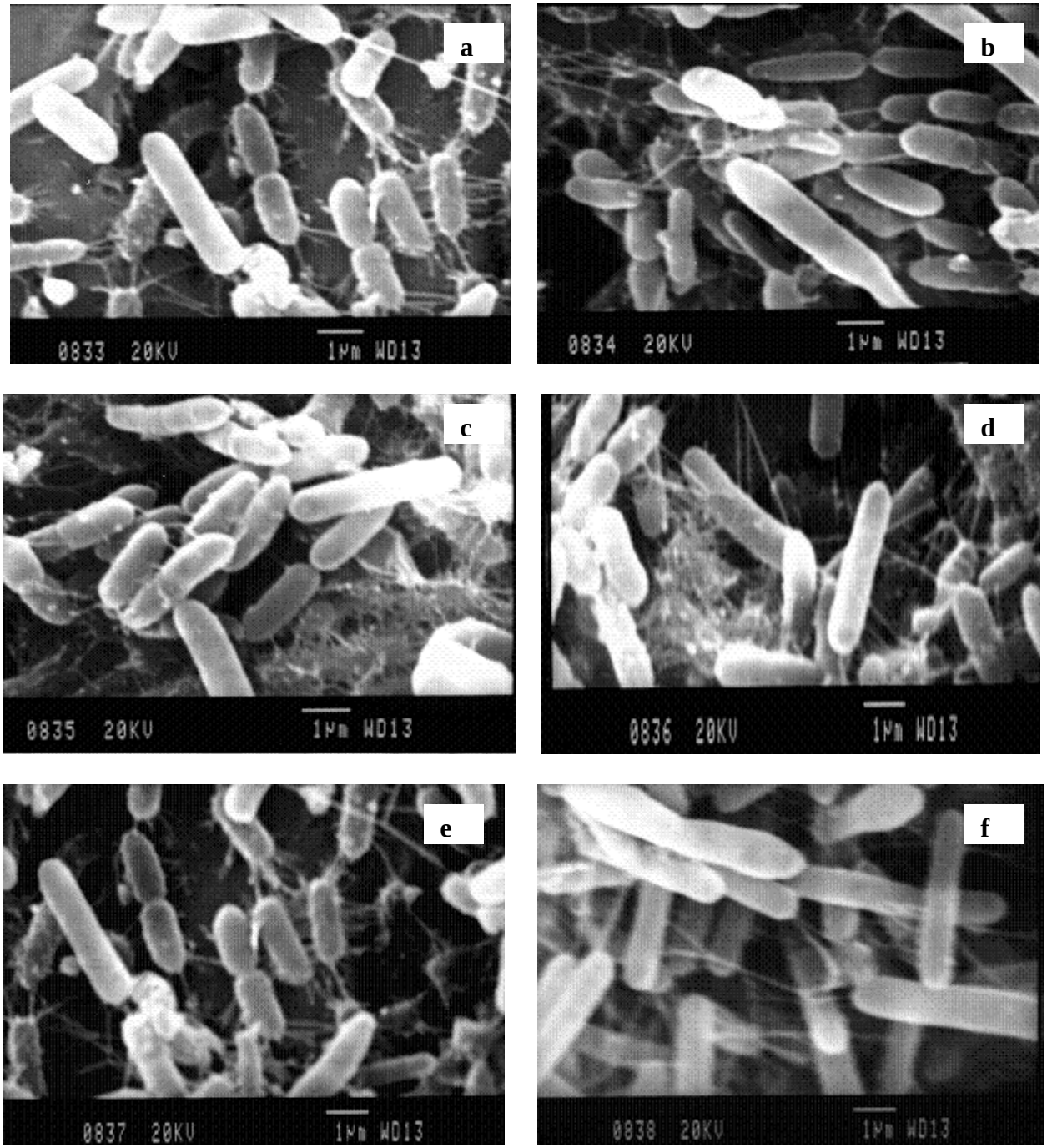

Figure 3.9 Scanning electron micrographs showing attachment of *Pseudomonas* species (BDS1, BDS2, GR1 and GR3FAR) to GAC in a fluidized bed biofilm bioreactor from batch culture experiments (a-f).

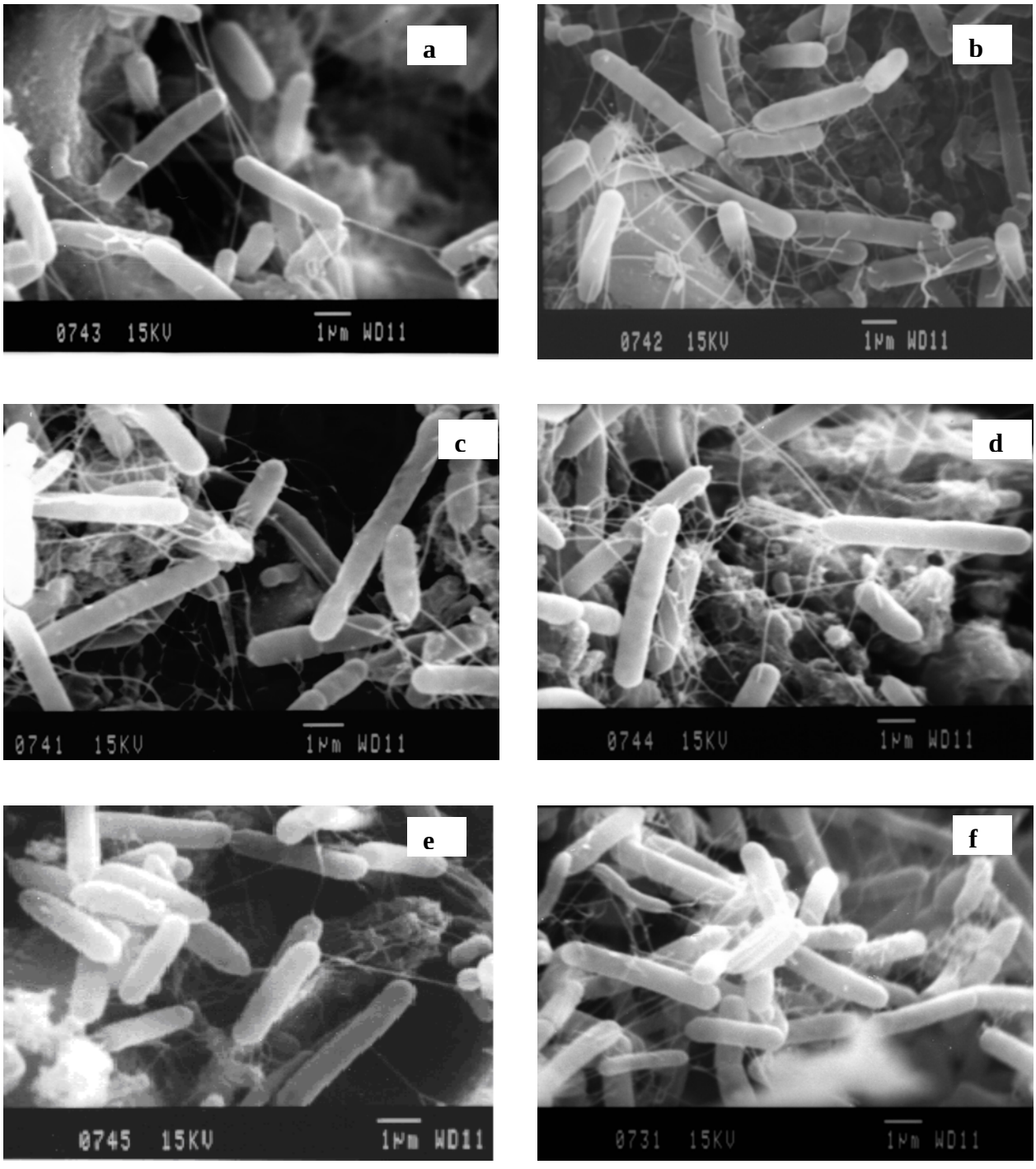

Figure 3.10 Scanning electron micrographs of co-cultures of two species of *Bacillus* (SYB4 and MAR) and four *Pseudomonas* species (BDS1, BDS2, GR1 and GR3FAR) attached to GAC in fluidized bed biofilm reactor from batch experiments (a-f).

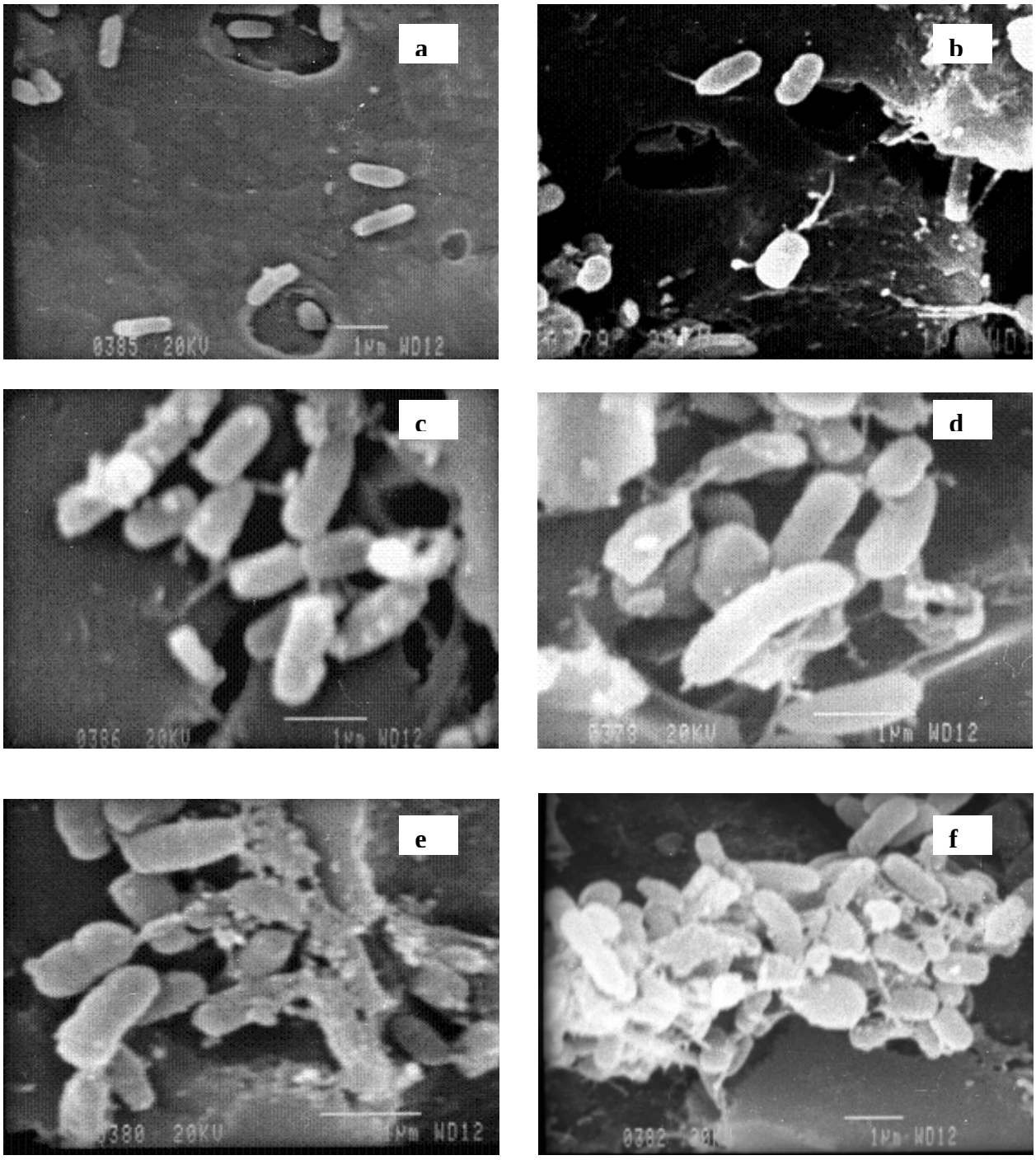

Figure 3.11 Scanning electron micrographs showing attachment of two *Bacillus* species (SBSY4 and MAR) on GAC in a fluidized bed biofilm bioreactor from control batch experiments (a-f).

Discussion

A variety of soil bacteria have been shown to possess the ability to degrade hydrocarbons, with *Pseudomonas* (Reardon *et al*., 2000) and *Bacillus* (Patil *et al*., 2006) being among the most important species (Leahy & Colwell, 1990; Chaillan *et al*., 2004). When these species were used either together or separately in this study, there was a measurable degradation of sodium benzoate, suggesting that they were capable of using sodium benzoate as the sole carbon and energy source (Patil *et al*., 2006). This was further confirmed by their ability to grow on BA plates indicating that they were capable of breaking down sodium benzoate suggesting that they had sodium benzoate – degrading enzyme system (Kimura & Ito, 2001).

Bacterial biomass and degradation of sodium benzoate by attached and freely suspended cells in batch cultures.

In this study, counts (log cfu/ml) of attached cells were compared to planktonic cells within bioreactors A and B, and to suspended cells from reactor C1. Degradation of sodium benzoate by biofilm cells from reactors A and B was compared to degradation by free cells from reactor C1 in one-way ANOVA. Differences in counts and degradations between reactors A, B and C1 were detected where data revealed significant ($P< 0.05$) differences through analysis of variance using a post ANOVA test (Bonferroni's multiple comparison test) to separate means.

As previously mentioned, the obtained results suggested that within reactors A and B, there were generally higher counts of attached cells than that of planktonic counts. Compared to suspended counts obtained from reactor C3, attached cells from reactors A and B, were still much higher. This was because attached cells are capable of surviving and persisting in microenvironments better than their planktonic counterparts (Sharanagouda & Karegoudar, 2002; Wang *et al*., 2004). Owing to this feature, immobilised cell systems are defined by high densities of active cells and good operational stability (Wang *et al*., 2004; Patil, *et al*., 2006). The reason for longer life span of attached cells compared to their freely living counterparts could be due to the fact that attached cells are reportedly protected either by EPS, if they are in biofilms (Singh *et al*., 2006**),** or by a more stable membrane if immobilized on matrices, like alginate beads (Patil, *et al*., 2006).

There was also more sodium benzoate degradation where biofilm cells were used. Suspended cells did not completely deplete sodium benzoate as there was never a point where the OD_{230} was zero. A similar observation was reported by Patil and colleagues (2006), who attributed incomplete degradation of phthalate by suspended cells to swelling of the cells (Patil, *et al*., 2006). The concentration of sodium benzoate used in this study was 10g/L which has been rated as high by other researchers **(**Sharanagouda & Karegoudar, 2002). Therefore at this concentration, freely suspended cells might have not coped well and this could have led to lower cell viability, explaining less degradation by suspended cells (Patil *et al*., 2006).

Similar results were obtained in a study done by Sharanagouda and Karegoudar (2002) where degradation of 2-methylnaphthalene by free and immobilized cells of *Pseudomonas* sp. was investigated. They reported that degradation by biofilm cells was much higher than by freely suspended cells at this concentration (Sharanagouda & Karegoudar, 2002). This suggested that biofilm cells were more efficient in degrading sodium benzoate compared to suspended cells.

Increased biodegradation of sodium benzoate when immobilized cells were used could also be attributed to immobilization-induced cellular or genetic modifications, such as increased ability to stand high substrate concentrations, which may favour substrate consumption (Zhu & Yang, 2003). Presence of attached cells to the matrices has also been reported to result in the alteration of physicochemical properties of the microenvironment. (Patil & Karegoudar, 2005), including the presence of ionic charges, reduced water activity, altered osmotic pressure, modified surface tension, and cell confinement (Zhu & Yang, 2003), all of which, result in higher microbial activity. High cell concentrations (Patil, *et al.,* 2006) and better survival (Singh *et al*., 2006) maintained by GAC attached cells over the corresponding suspended cells, also lead to increased rates of reactions, which would also explain higher degradation by attached cells as compared to suspended cells (Sharanagouda & Karegoudar, 2002; Patil & Karegoudar, 2005; Patil, *et al.,* 2006).

Sodium benzoate degradation was faster in experiments that maintained higher bacterial counts (reactors A and B), i.e., as cell populations increased, the amount of sodium benzoate in FBBR decreased, suggesting that more cell growth resulted in more substrate consumption (Qureshi *et al*., 2005). A similar trend was observed in a study done by Amsden and colleagues (2003) on phenol degradation by *P. putida* in a partitioning bioreactor, who reported a decrease in phenol concentration and a concurrent increase in cell concentration (Amsden *et al*., 2003).

Reportedly, techniques used for monitoring bacterial metabolic activity include the use of extracellular enzymatic activity measurements (Boetius & Lochte, 1994), radiolabeled amino acids incorporation (Alongi, 1990) and the direct viable count method (Kogure *et al.*, 1979). This confirms that high metabolic activity is associated with higher plate counts. Therefore, higher degradation rates in these bioreactors could without any doubt be attributed to the higher cell densities (Shim & Yang, 1999).

Operation of bioreators as continuous systems

Continuous culture systems are described as steady-state systems where a constant supply of nutrient and a steady amount of biomass is maintained in the bioreactor (Mkandawire *et al*., 2005). The principal advantage of continuous culture systems is that the rate of dilution controls the rate of microbial growth (Hoskisson & Hobbs, 2005).

After FBBR operation under batch conditions for 20 days, the operation was changed to the continuous system. Under the continuous mode, the bioreactor was run at five different flow rates, 25ml/min, 85ml/min, 100 ml/min, 140ml/min and 170ml/min for 5 days.

The observation that degradation increased with increasing flow rate but only when the flow rate was below 170ml/min, above which microbial degradation capacity dropped correlated with the results reported in a study done by Sharanagouda and Karegoudar, (2002) on degradation of 2-methylnaphthalene by of *Pseudomonas* sp. strain NGK1 (Sharanagouda & Karegoudar, 2002).

The decrease in the degradation of sodium benzoate at the highest flow rate (170ml/min) could be due to insufficient mass transfer (Mordocco *et al*., 1999), slow diffusion of the substrate in biofilm (Liu *et* al., 2005) or short contact time between substrate and attached colonies which formed a biofilm (Abou Seoud & Maachi, 2003). These factors have been shown to decrease degradation rates mostly at high substrate concentrations (>50mM) (Sharanagouda & Karegoudar, 2002). As previously stated, 10g/L of sodium benzoate was used throughout, i.e. 69.4 mM, which would explain why there was less degradation at the highest flow rate.

Mass transfer

Mass transfer can be defined as transportation of substrate, oxygen and other nutrients from the bulk liquid across the stationary boundary layer of the biofilm and then across the biofilm surface boundary (Vinod & Reddy, 2006). This diffusion affects the stability and structure of the biofilm (Liu *et al*., 2005). Even though fluidized bed bioreactors have been reported to result in efficient mass transfer (Abou Seoud & Maachi, 2003) and large surface area for biofilm formation (Sokol & Korpal, 2006); insufficient fluidization could still lead to mass transfer resistance (Abou Seoud & Maachi, 2003). In aerobic systems, the rate at which oxygen is transferred to cells is one of the most important limiting factors for bioremediation of organic compounds (Dhanasekharan *et al*., *2005*).

Degradation of sodium benzoate in reactors A, B and C3

In all the experiments (batch and continuous), there was more degradation in reactor A where only the Gram-negative consortium was used, compared to reactor C3, where only suspended Gram-positive species were used. This observation could be explained by reports that unlike Gram-negative bacteria, Gram-positive bacterial growth and degradation abilities are reduced by high concentrations of aromatic hydrocarbons such as 2,4,6-trinitrotoluene (TNT) (Fuller & Manning, 1997). The difference in Gram-negative and Gram-positive bacterial response to certain compounds could be attributed to the differences in their cell wall structure (Dahl *et al*., 1989). Gram-negative bacteria

have the outer membrane, which has been reported to prevent entry of aromatics into the cells. On the other hand, Gram-positive bacterial cell walls may be permeable to these aromatics (Fuller & Manning, 1997), as they lack the outer protective structure (Dahl *et al*., 1989).

Similarly, more degradation was observed in reactor B where co-cultures of Gram-negative and Gram-positive bacteria were used compared to reactor C3. This could be attributed to improved microbial activity and stability of mixed cultures compared single cultures (Ambujom, 2001).That is, mixed cultures withstand unfavourable conditions better compared to single cultures and pure cultures. These results are in agreement with results obtained in a study done Kimura & Ito (2001), which investigated Terephthalic acid (TPA) using mixed culture systems of bacterial strains C4S (Gram-negative aerobic rod) which was identified as a *Pseudomonas* sp. and C4B (Gram-positive aerobic rod) which was identified as *Bacillus brevis*. Their results demonstrated increased TPA degradation when mixed C4S and C4B were used (Kimura & Ito, 2001).

Comparison of degradation of sodium benzoate in batch and continuous configurations.

The findings that there was more degradation when reactors were operated under the batch mode compared to the corresponding biofilm configuration operated using the continuous system could be attributed to reduced inhibitory effects exhibited by batch

cultures (Bali & Sengul, 2002) Reportedly, unstable conditions such as exposure time that are associated with reactor operation, can easily be controlled under batch and, as a result, highly active bacterial populations dominate (Mohan *et al*., 2007a). Batch cultures also result in constant circulation of the biomass leading to increased resistance to substrate shock (Kaballo *et al*., 1995), on the other hand, continuous operations have been reported to have limited activity due to uneven biomass distribution (Kaballo *et al*., 1995; Mohan *et al*., 2007b). Similar results were observed in a study done by Cassidy and Irvine, (1997) in degradation of diesel fuel. They reported higher degradation in batch operation conditions compared to the continuous operation (Cassidy & Irvine, 1997). In another study which investigated treatment of combined mixture (composite) of wastewater, higher removal efficiency was also reported in batch mode compared to the continuous mode (Mohan *et al*., 2007a). Continuous mode of operation has also been reported to have higher biomass production but decreased overall degradation abilities (Reumerman, 2004).

The higher bacterial populations observed in the continuous culture mode, could be due to the fact that under such growth conditions, the rate of bacterial growth (or doubling of bacterial numbers) increases in direct proportion to the rate at which fresh nutrient (effluent) is added to the system (Mkandawire *et al*., 2005).

Scanning electron microscopy (SEM)

Cells in a biofilm can be differentiated from their suspended counterparts by, among other things, the production of an extracellular polymeric substance (EPS) matrix (Bhinu, 2005). Biofilms have defined architecture (Donlan, 2002), which plays a very important role in microbial behaviour (Donlan, 2002; Guiot *et al*., 2002). Therefore, the study of biofilm architecture is very important. However, the constituent bacterial cells forming the biofilm are very small, so only microscopes with a high resolution would be used to study or view biofilm morphology (Guiot *et al*., 2002). SEM has been successfully used to view biofilm morphology and arrangement (Davey & O'toole**,** 2000; Wimpenny *et al*., 2000).

Scanning electron micrographs showed there was a general attachment of cells to GAC surfaces. Most cells had a normal short-rod shape which was not different from the normal rod-shaped morphology which is characteristic of both *Pseudomonas* and *Bacillus* species (Shim & Yang, 1999). In addition, scanning electron micrographs showed that some cells occurred singly or in pairs as short rods (figures 3.7a, e, 3.9a and b) – a normal morphology shown by *Pseudomonas* in liquid culture (Buchanan & Gibbons, 1993).

These findings meant that some cells exhibited no morphological differences from normal morphology. This suggested that the cells had been grown under nutrient-rich conditions as stress induced morphology changes have been reported to result from

nutrient deprivation (Jan *et al*., 2001; Allan *et al*., 2002). A trace element solution, organic nitrogen source and other nutrients were provided in the medium (Shim & Yang, 2002). The normal rod shaped morphology maintained by most cells can be attributed to the good growth conditions imposed by operating conditions used in this study. However, even better results could have been obtained if, in addition to aeration, additional oxygen (as provided by H_2O_2) was provided to the column (Shim & Yang, 2002).

However, a close observation of cells has shown that some cells from reactors A and B exhibited morphological changes such as indentations and elongation. This could have been caused by either nutrient deprivation as motioned above (Sternberger *et al*., 2002) or adaptation of the bacteria to the bioreactor environment signals like the presence of toxins (Shim & Yang, 1999). During batch experiments, nutrients are recycled and with time some nutrients could be depleted. Reportedly, elongation is observed in *Pseudomonas* species that are in biofilms, but not in solution (Sternberger *et al*., 2002).

The obvious filamentous bacteria observed in the micrographs, that may have been *Pseudomonas*, could have resulted from oxygen limitation (Allan *et al*., 2002). The same filamentous morphology of *Pseudomonas* cells in biofilms was observed by McCoy and Costerton in 1982 (McCoy & Costerton, 1982) but in their case, it was as a result of starvation.

Conclusion

Isolated *Pseudomonas* species – *P. aeruginosa* BDS1 (Accession number EU096503), *P. putida* BDS2 (Accession number EU096504), *P. putida* GR1 (Accession number EU111745) and *P. putida* GR3FAR (Accession number EU103614) used to seed a laboratory-scale fluidized bed biofilm bioreactor, degraded sodium benzoate successfully and hence may be useful in bioremediation of other aromatic pollutants.

Batch systems were more efficient in bioremediation of sodium benzoate compared to the continuous system. Compared to biodegradation by planktonic cells, biofilm-mediated bioremediation, regardless of whether the biofilm consisted of only *Pseudomonas* species or mixtures of different species in FBBR, provides an effective way for elimination of ring-containing organic pollutants.

Attached cells had a better chance of survival and survived for longer periods. This was shown by attached cells dominating in the FBBR compared to freely suspended cells. The concentration or the number of viable cells in FBBR determined the rate at which microorganisms utilized sodium benzoate, where more cells lead to a higher sodium benzoate bioremediation.

Pseudomonas isolates easily formed multi-species biofilms in laboratory-scale fluidized bed biofilm bioreactors. This was shown by scanning electron microscopy.

This study confirmed that FBBR technology is efficient in treating wastewater containing sodium benzoate.

Chapter 4

Summarizing discussion and conclusion

Background

Releasing untreated wastewaters from industries to the environment does not only harm the environment (Ramos *et al*., 2007) but could also endanger aquatic life (Üstün *et al*., 2007). For example dyes have been reported to be toxic to fish (Lin & Peng, 1996; Kim *et al*., 2004). Some of the industrial aqueous effluents contain toxic hydrocarbons, like phenol, which are also a thread to human health (Tziotzios *et al*., 2005). Therefore, it is very important to treat wastewater before discharging it into the environment. Other conventional treatments that have been used for treating toxic aromatic containing wastewaters, such as coagulation and adsorption (Ochieng *et al*., 2003), are very expensive (Aslam *et al*., 2007) compared to biological methods, such as fluidized bed biofilm reactors (Ochieng *et al*., 2003).

Biofilm bioreactors are in general more efficient than other types of reactors (Qureshi *et al*., 2005) because they maintain higher biomass concentration (Zilouei, *et al*., 2006). Of all biofilm reactors, fluidized bed bioreators are the most popular (Tang *et al*., 1987; García Encina & Hidalgo, 2005) and have been preferred over other methods in the treatment of toxic compounds (Ochieng *et al*., 2003) since they offer advantages (Zilouei, *et al*., 2006), such as higher bioremediation capacity (Heijnen *et al*., 1989; Ochieng *et al*., 2003; Qureshi *et al*., 2005), and even distribution of the liquid phase bringing about phase homogeneity (Saravanane & Murthy, 2000; Ochieng *et al*., 2003), which gives them the ability to operate efficiently even at high volumes.

Selection of experimental parameters for this study

The purpose of this study was to use an aerobic fluidized bed biofilm reactor for the treatment of wastewater containing benzoate.

Four *Pseudomonas* species were used in this study because *Pseudomonas* species have been shown to have the ability to degrade recalcitrant aromatics, with *Pseudomonas putida* being the most studied species (Reardon *et al*., 2000). The isolated species that were used were identified as *Pseudomonas putida* BDS1 (Accession number EU096503), *Pseudomonas aeruginosa* BDS2 (Accession number EU096504), *Pseudomonas putida* GR1 (Accession number EU111745) and *Burkholderia cepacia* GR3FAR (Accession number EU103614). Two *Bacillus* species *Bacillus macroides* SBSY4 (Accession number EU096501) and *Bacillus simplex* MAR (Accession number EU111744) were also used in this study as *Bacillus* has also been used in degradation of aromatics such as *o*-phthalate (Patil *et al*., 2006).

Fluidized bed biofilm reactors (FBBRs) were used in this study because of many advantages they offer compared to other biofilm reactors. For example, they have a higher biomass concentration (Lazarova & Manem, 1995) higher surface area (Bohlmann & Bohner, 2001), an increased contact between the solid and the liquid phase making liquid phase homogeneity possible of the liquid (Ochieng *et* al., 2003). Owing to all these advantages, FBBRs are also known for their increased microbial activity (Saravanane, &

Murthy, 2000) resulting in high performance (Tang *et al*., 1987). In addition, FBBRs require small operational space (Heijnen *et al*., 1989).

Consortia of defined species (BDS1, BDS2, GR1, GR3FAR, SBSY4 and MAR) that have the ability to degrade sodium benzoate were used in this study. Numerous studies on biodegradation of organic compounds, such as phenol, only used pure cultures (Chen *et al*., 2002; Patil *et al*., 2006). Just a few studies have used mixed cultures (Tziotzios *et al*, 2005; Lindsay *et al*., in press). Of those few utilizing mixed cultures, most were undefined or uncharacterized consortia (Ambujom, 2001). Therefore, this was the first study to utilize a defined mixed culture in FBBR, under batch and continuous conditions. However, it is vital to use defined consortia that are known to degrade the organic compound of interest as that could lead to increased biodegradation rates as biodegradation depends on the ability of individual species that make up the consortium to degrade the substrate (Ambujom, 2001).

Granular activated carbon (GAC) was used as the support medium. GAC as a biofilm carrier in reactor system, has been reported to have high adsorptive capacity leading to reduced pollutant concentrations (Carvalho *et al*., 2001), a rough surface that provides a good niche for bacterial colonization and protection from fluid forces (Herzberg *et al*., 2005) and increased bacterial attachment resulting from the presence of variety functional groups found on GAC surfaces (Weber *et al*., 1979). Furthermore, bacterial populations used in biofilm reactors where GAC is used as a support medium have been found to have elevated activity attributed to substrate flux form both GAC solid phase and the bulk phase (Herzberg *et al* ., 2005).

Three control experiments were conducted in this study. 1) To allow for comparisons of degradation by suspended cells and GAC attached cells, reactors without GAC were conducted. 2) To confirm that the observed degradations as shown by OD_{230} readings were not due to other degradation mechanisms such at photo degradation, control reactors without the inoculum were performed. 3) Finally, to allow for comparisons of degradation by Gram-negative and Gram-positive, reactors with only Gram-positive inoculum were operated.

To quantify cell density of both attached and planktonic cells, plate counts were used. Cells were on grown on NA and BA. Attached cells were dislodged from GAC surfaces by shaking with glass beads before 10 min before plating (Lindsay *et al.*, 2002; Lindsay *et al*., in press). SEM was used to view cells attached on GAC. The use of SEM allowed the visualization of dehydrated specimens at relatively high resolution and provided useful information on bacterial morphology (Guiot *et al*., 2002). It also defined the number and distribution of bacterial populations attached to surfaces (Austin & Bergeron, 1995).

Chapter 2

Isolation and identification of novel benzoate-degrading bacterial populations

Chapter 2 involved isolation and identification of benzoate-degrading microbial populations. Bacteria were isolated from industrial soils exposed to continual diesel or petrol spillage. After isolation, DNA was extracted using the boiling method (Lindsay *et al*., in press). Then PCR and sequencing followed. Then a phylogenetic tree was constructed using the neighbour joining method.

Only populations with the ability to degrade hydrocarbons were selected for in this study. This was demonstrated by colonies forming on agar plates containing a variety of hydrocarbons including toluene, butanol and sodium benzoate. Reportedly, growth in media containing hydrocarbons means that the isolated bacterial populations had the ability to degrade the hydrocarbons (Okerentugba & Ezeronye, 2003).

Isolated species clustered within previously isolated hydrocarbon degraders such as *P. aeruginosa* CS1CO (DQ304683) and *P. putida* DSS2 (DQ304685) (Lindsay *et al*., in press).

Chapter 3

Biodegradation of sodium benzoate in a fluidized bed biofilm reactor (FBBR)

To evaluate microbial activity in FBBR, sodium benzoate degrading populations isolated and identified in chapter 2, were used to inoculate the reactors. In this study, two experiments were conducted. One experiment involved the used of four *Pseudomonas* species while the other one used the mixture of four *Pseudomonas* species and two *Bacillus* species. Reactors were initially operated as batch systems for 20 days after which the operation was changed to the continuous mode. The continuous system was operated for 5 days at different dilution rates including 25ml/min, 85ml/min, 100ml/min, 140ml/min and 170ml/min. Cell density was quantified by plating on NA and BA. Sodium benzoate degradation was measured at 230nm.

In keeping with literature reports (Sharanagouda & Karegoudar, 2002; Wang *et al*., 2004; Patil, *et al*., 2006) counts of GAC attached cells were higher than freely suspended cells in FBBR under both batch and continuous configurations. There was also higher sodium benzoate degradation associated with attached cells compared to freely suspended cells. This suggested that higher biomass concentrations lead to increased degradation. Reportedly, biofilm reactors using GAC immobilized cells show increased microbial activity compared to reactors using suspended cells (Herzberg *et al.*, 2005).

Conclusions

1. Strains of *P. aeruginosa, P. putida, B. simplex* and *B. macroides* were isolated as possible bioremediation organisms of sodium benzoate.

2. The use of both mixed cultures of Gram-negative and Gram-positive and Gram-negative only resulted in more degradation compared to where only Gram-negative bacteria were used.

3. GAC attached microbial populations were more effective in degrading sodium benzoate compared to suspended cells.

4. Sodium benzoate degradation was achieved when reactors were operated under both batch and continuous modes and in both cases the major determinant of the rate of degradation was cell counts (log cfu/ml). However batch systems were more efficient in bioremediation of sodium benzoate.

5. Overall, sodium benzoate was successfully degraded in a laboratory-scale fluidized bed biofilm bioreactor using the isolated strains and immobilization of bacteria is the basis for the use of this technology.

6. Scanning electron microscopy indicated that all strains formed biofilms.

Significance of the present study

Biological waste water treatment is very efficient and bacteria with the ability to degrade a various hydrocarbons are the key to the success of biological systems. However, the importance of wastewater treatment in bioreactors using such bacterial populations is normally neglected in research. This study therefore provides insight to how bacterial growth is affected by hydrocarbons and if there is any relationship between the active biomass and biodegradation. This information could be used in the development of aerobic biofilm reactors for effective biological wastewater treatment.

In this work, PCR targeting the 16S rDNA and plate counts were successfully used to study microbial diversity and quantity respectively. It is very important to use accurate techniques in isolating and quantifying bacterial populations because a better understanding of the microbial diversity and quantification of viable biomass would provide information on mechanics of industrial wastewater treatment unit and eventually aid in achieving higher reactor performance.

The mode of operation is one of the key parameters in the optimization of bioreactor performance. The FBBR system used in this study was operated under the batch and continuous modes. Each of these modes of operation, have advantages and disadvantages with respect to stability and performance, therefore operating reactors under these two modes would lead to improved reactor performance. This study also gives details on how

different flow rates may affect bacterial cell growth, hence, bioremediation of hydrocarbons.

As mentioned earlier, FBBRs operational performance allows for reduced space requirements. This would be particularly advantageous for treatment of wastes from industries that very often have space limitations and produce large amounts of wastewaters.

Considerations for future work

Many bioreactor technologies have been used for degradation of hydrocarbons in wastewaters. It has been shown that the efficacy of the bioreactors used in bioremediation is affected by physiochemical factors such as pH, temperature, nutrients, oxygen transfer and the concentration of the hydrocarbon (Abou Seoud & Maachi, 2003). It would therefore be very important to carry out experiments investigating the effects of these variables on biodegradation of aromatics. This way, the variables could be controlled and conditions optimized in such a way that the highest microbial activity, hence the highest biodegradation rates are achieved.

A limitation in this study was that only SEM was used to visualize bacterial morphology. Although this technique provides information on bacterial adhesion, other alternatives for studying hydrated microbial cells and cell viability, such as confocal scanning laser microscopy (CSLM) could have been used as well.

Another limitation in this study was that, a simple structured benzoate was used as a hydrocarbon for investigation. Most industrial waters contain more complex hydrocarbons such as phenol (Aksu & Yener, 2001; Dabrowski *et al*., 2005) and toluene (Gómez *et al*., 2001). Therefore future studies using such aromatic compounds should be considered.

Chapter 5

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Chapter 6

Appendix

Sequences of *Pseudomonas* isolates

Isolate SBSY5 (EU096502):

**GGATGAAGAGAGCTTGCTCTCTGATTCAGCGGCGGACGGGTGAGTA
ATGCCTAGGAATCT
GCCTGATAGTGGGGGACAACGTTTCGAAAGGAACGCTAATACCGCA
TACGTCCTACGGGA
GAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGT
CGGATTAGCTAGTT
GGTGAGGTAACGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAG
AGGATGATCAGTCA
CACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTG
GGGAATATTGGACA
ATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCT
TCGG**

Isolate BDS1 (EU096503):

**CCTAACACTGACAATCTAGCGGTAGACGGGAGCTTGCTCCTTGATT
CAGCGGCGGACGGG
TGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGACAACGTTTCG
AAAGGAACGCTAAT
ACCGCATACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGC
GCTATCAGATGAGC
CTAGGTCGGATTAGCTAGTTGGTGGGGTAATGGCTCACCAAGGCGA
CGATCCGTAACTGG
TCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGAC
TCCTACGGGAGGCA
GCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATG
CCGC**

Isolate BDS2 (EU096504):

**CCCAGCGAGAAGGGAGCTTGCTCCTGGATTCAGCGGCGGACGGGT
GAGTAATGCCTAGGA
ATCTGCCTGGTAGTGGGGGATAACGTCCGGAAACGGGCGCTAATAC
CGCATACGTCCTGA
GGGAGAAAGTGGGGGATCTTCGGACCTCACGCTATCAGATGAGCCT
AGGTCGGATTAGCT
AGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCGTAACTGGTC
TGAGAGGATGATCA
GTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGC
AGTGGGGAATATTG
GACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAA
GGTCTT**

Isolate GR1 (EU111745):

**CTAGGATGACGGGAGCTTGCTCCTTGATTCAGCGGCGGACGGGTGA
GTAATGCCTAGGAA
TCTGCCTGGTAGTGGGGGACAACGTTTCGAAAGGAACGCTAATACC
GCATACGTCCTACG
GGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTA
GGTCGGATTAGCTA
GTTGGTGGGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCT
GAGAGGATGATCAG
TCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCA
GTGGGGAATATTGG**

Isolate GR3FAR (EU103614):

**GTCTGAGCGGATGAAGGGAGCTTGCTCCTGGATTCAGCGGCGGAC
GGGTGAGTAATGCCT
AGGAATCTGCCTGGTAGTGGGGGACAACGTTTCGAAAGGAACGCTA
ATACCGCATACGTC
CTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGA
GCCTAGGTCGGATT
AGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAACT
GGTCTGAGAGGATG
ATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGG
CAGCAGTGGGGAAT
ATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGA
AGAAGGTCTTCGGA**

Isolate FOR (EU103615):

**TCTGAGCGGTAGAGAGAAGCTTGCTTCTCTTGAGAGCGGCGGACGG
GTGAGTAATGCCTA
GGAATCTGCCTGGTAGTGGGGGATAACGTCCGGAAACGGACGCTAA
TACCGCATACGTCC
TACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAG
CCTAGGTCGGATTA
GCTAGTTGGTGGGGTAATGGCTCACCAAGGCGACGATCCGTAACTG
GTCTGAGAGGATGA
TCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGC
AGCAGTGGGGAATA
TTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAA
GAAG**

Isolate FAR1 (EU103616):

**CATGCGAGTCGACGCGGATGAGAGGAGCTTGCTCCTGGATTCAGCG
GCGGACGGGTGAGT
AATGCCTAGGAATCTGCCTGGTAGTGGGGGACAACGTTTCGAAAGG
AACGCTAATACCGC
ATACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTAT
CAGATGAGCCTATG
TCGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATC
CGTAACTGGTCTGA
GAGGATGATCAGGCCCACTGGAACTGAGACACGGTCCAGACTCCTA
CGGGAGGCAAAGTG
GGGAAAGGACAAGGGCGAAAGCCTGATCCAGCCATGCCGCGGTGT
GTGAA**

Sequences of isolated *Bacillus* species

Isolate SBYS4 (EU096501):

**GCATAATACATGCAAGTTTGAGCGAATCGATGGGAGCTTGCTCCCT
GAGATTAGCGGCGG
ACGGGTGAGTAACACGTGGGCAACCTGCCTATAAGACTGGGATAAC
TTCGGGAAACCGGA
GCTAATACCGGATACGTTCTTTTCTCGCATGAGAGAAGATGGAAAG
ACGGTTTACGCTGT
CACTTATAGATGGGCCGCGCGGCGCATTAGCTAGTTGGTGAGGTAA
TGGCTCACCAAGGC
GACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGAC
TGAGACACGGCCCAG
ACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAA
GTCTGA**

Isolate MAR (EU111744):

**AGCCTGAGCGAATCGACGGGAGCTTGCTCCCTGAGATTAGCGGCGG
ACGGGTGAGTAACA
CGTGGGCAACCTGCCTATAAGACTGGGATAACTTCGGGAAACCGGA
GCTAATACCGGATA
CGTTCTTTTCTCGCATGAGAGAAGATGGAAAGACGGTTTACGCTGT
CACTTATAGATGGG
CCCGCGGCGCATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCG
ACGATGCGTAGCCG
ACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAG
ACTCCTACGGGAGGC
AGCAGTAGGGA**